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Equipment for the Resuspension or Redissolving of Precipitates in Continuous-Flow Centrifuges

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Summary. Equipment has been developed for resuspending or redissolving protein precipitates separated in a continuous-flow centrifuge without first removing them from the centrifuge bowl. All the components can be sterilised by autoclaving. The equipment has been used successfully in selected stages of a process for the preparation of human plasma fractions.

Introduction

Human gamma globulin, albumin and other protein constituents of human blood plasma are prepared on a large scale by precipitation of the various fractions with organic solvents under controlled conditions of temperature, ionic strength, pH and protein concentration. Precipitates are usually collected by centrifugation and a continuous-flow centrifuge of the Sharples type is frequently used for this purpose. For further purification or for making the final solution, it is necessary: (1) to remove the precipitate from the centrifuge bowl, and (2) to bring it into solution or suspension in a suitable mixing vessel. This is generally carried out in two stages which are time-consuming and difficult to perform aseptically.

When a continuous-flow centrifuge was installed at this laboratory for use at certain stages in the fractionation of human plasma by precipitation with ethyl ether by the method of Kekwick and Mackay (1954), a method of processing precipitates in one stage after centrifuging was developed so as to maintain the closed method of working that is a feature of this technique of fractionation. The equipment and its operation are described in this paper.

Equipment

By means of special fittings attached to the centrifuge bowl* after removing it from the centrifuge, the solution in which a precipitate is to be dissolved is circulated through the bowl with stirring until complete solution is achieved. All parts of the fittings that are in contact with the solution are made of stainless steel, excepting glands and seals. Materials suitable for sterilisation by autoclaving are used throughout.

(*a*) *The Agitator Assembly*

The agitator consists of two strips of stainless steel, 0·625 in. wide, mounted helically on a $\frac{3}{4}$-in. shaft of length 20 in. extending almost the full length of the cylindrical bowl (Fig. 1). The shaft

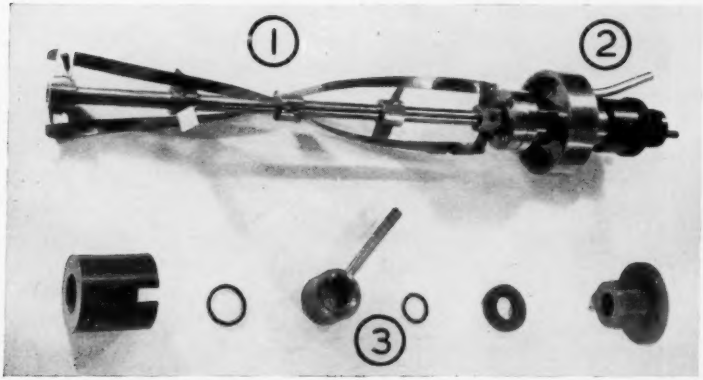

Fig. 1. Fittings for attachment to the centrifuge bowl to resuspend precipitates. (1) Agitator, (2) cap with shaft seal and bearing housings and outlet tube, and (3) inlet tube and collar with ring seals and clamp

is fitted centrally on a metal cap which is threaded internally so that it can be screwed onto the end of the Sharples bowl in place of the cap used when centrifuging. When in position, the shaft rotates coaxially in the bowl with a clearance of 0·2 in. between the outer edges of the strips and the inner surface of the bowl. The cap carries a shaft seal (Crane type 109) and a bearing

* Sharples No. 6 Presurtite Supercentrifuge.

housing for phosphor bronze sleeve bearings. At one end of the shaft is a coupling to connect with a motor when it is in the running position. To produce turbulence in the bowl, two sets of small blades with pitch opposite to that of the helical strips are mounted on the shaft.

A $\frac{1}{2}$ in. bore tube, let into the cap at one side, acts as an outlet for the solution in which the precipitate is to be dissolved.

(*b*) *The Inlet Attachment*

At the end of the bowl remote from the cap, a fitting is connected to the discharge holes to provide an inlet for the solution (Figs. 1 and 2). It consists of a stainless steel collar with an

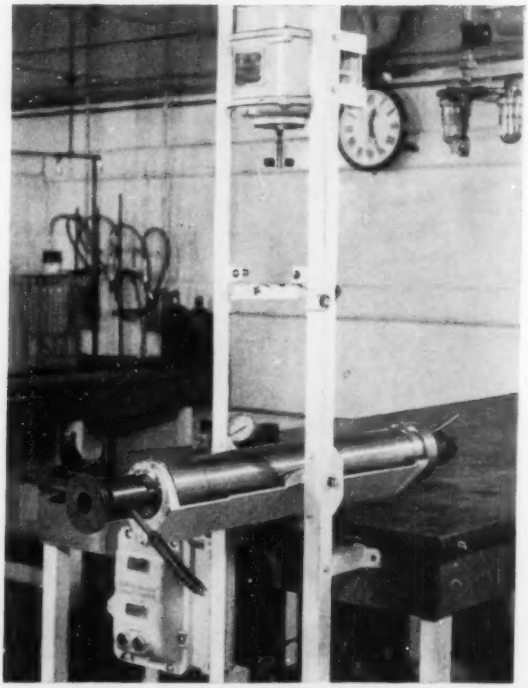

Fig. 2. The centrifuge bowl with its carrier in the horizontal position. The agitator assembly and inlet attachment are fitted

annular cavity which fits over the holes. A $\frac{1}{2}$ in. bore side tube opens into the cavity. The ends of the collar are bevelled so that neoprene ring-seals can be compressed onto the neck of the bowl by tightening a cylindrical clamp, thus securing the collar in position. The clamp is of brass and has a slot to provide clearance for the side tube.

(*c*) *The Carrier and Motor*

A carrier is provided to hold the bowl while the agitator assembly and inlet attachment are being fitted and while the agitator is running (Figs. 2 and 3). It swings on trunnions at its

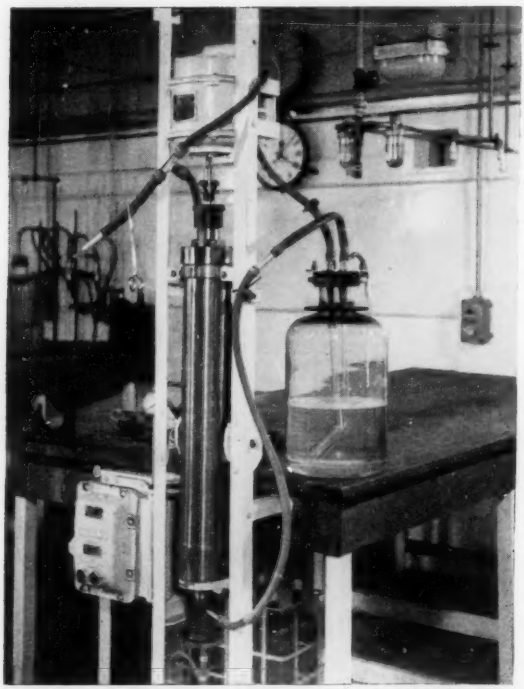

Fig. 3. The centrifuge bowl with its carrier in the vertical position. The inlet and outlet tubes are connected to the vessel containing fluid in which the precipitate in the bowl is to be resuspended

point of balance so that the bowl can be held horizontally while the fittings are attached and then be raised to a vertical position under the motor ($\frac{1}{4}$ h.p.: 1400 rev/min). The coupling on the shaft then engages with a coupling on the motor, making a simple form of universal joint.

Operation

The centrifuge bowl containing a precipitate is placed on the carrier in a horizontal position (Fig. 2). The cap is removed and, if a three-wing insert has been used in the bowl, it is withdrawn. The agitator is inserted in the bowl and the retaining cap is screwed into position. The cover used to protect the other end of the bowl while taking it from the centrifuge is now removed and the inlet attachment put in position. The bowl on its carrier is then swung to the vertical and clamped (Fig. 3). The side tubes are connected to a reservoir containing the solution to be circulated. The inlet tube on the reservoir is set tangentially so that the inflow causes the contents to swirl. The outlet tube reaches to the bottom of the reservoir from which solution returns to the bowl. The reservoir is filled initially with the volume required to dissolve the precipitate. The volume should be sufficient at least to fill the bowl, leaving the orifice of the outlet tube of the reservoir covered, otherwise the system will become airlocked. Solution is forced by compressed air into the centrifuge bowl until it is full. The agitator is tested for freedom of rotation. If the precipitate is bulky, the agitator blades will be embedded in it and the agitator is worked free by hand until it can make a complete rotation. The motor is then switched on. Having a propeller-like action, the agitator causes a flow of solution from the reservoir, up through the bowl and back to the reservoir. Suspension can be obtained more rapidly if, after switching on the motor and checking that circulation is taking place, the tube to the reservoir is closed for a few minutes. By this procedure, the precipitate is first broken down and mixed in the small volume of solvent in the bowl, after which the return tube is reopened so that the solution can circulate. The agitator is left running until a uniform suspension is obtained, usually between 15 min and 1 h. The degree of agitation is greater than

is usual in stirring plasma protein solutions. This is possible because air has been displaced from the bowl, and frothing cannot occur. After switching off the agitator, the residual solution in the bowl is drained into the reservoir, which is placed at a lower level for this purpose. Drainage can be hastened by applying compressed air to the bowl.

For protection when not in use, the agitator is kept in a cylindrical brass canister which has a rim threaded to fit the cap. The unit is autoclaved in the canister in a vertical position. During autoclaving or when not in use, it is kept vertical to prevent loss of oil from the bearing housing.

Application to the Fractionation Process

In fractionation by the method of Kekwick and Mackay (1954), the equipment has been successfully used for the separation of fractions F_1, G_1, AP and AP_2 working with pools of plasma of 60 l. Precipitate paste up to 4 l. in volume is recovered in the centrifuge bowl.

In the preparation of fibrinogen, fraction F_1 is collected as a precipitate by centrifuging and washed in a solution of ether in citrate saline. For the washing, the equipment is run until the precipitate is completely resuspended. The precipitate is separated by centrifuging again, and finally dissolved by circulating citrate saline through the bowl. Fraction AP, crude albumin, is treated similarly, water being used to take up the precipitate. The insoluble protein is centrifuged out, leaving a solution of albumin for further purification. The other fractions named are separated in the centrifuge as precipitates and redissolved by circulating the appropriate solvent.

Two sets of the equipment have been in use with a Sharples No. 6 centrifuge for over two years, and have only required routine maintenance. The yields of gamma globulin and albumin are as good as those obtained by bottle centrifugation of precipitates and resuspension in individual bottles, and of the same purity. The yield of fibrinogen is lower by 15 per cent with a slight increase in purity, possibly because resuspension at the washing stage is more thorough. The equipment has therefore not been used routinely for the separation of fibrinogen.

Conclusion

The equipment described makes it possible to resuspend or to dissolve precipitates collected in the bowl of a continuous-flow centrifuge without first having to remove them from the bowl. It has been successfully used at various stages in a method of fractionating human plasma proteins and may have similar applications in other chemical and biochemical procedures in which the sequence of centrifuging and resuspending or dissolving precipitates occurs.

Acknowledgement. My thanks are due to Mr. B. Smith, A.I.S.T., and Mr. W. J. Wilson for their help in the development of this equipment.

Reference

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Oxygen Transfer in Fermentation Systems—I. Use of Gluconic Acid Fermentation for Determination of Instantaneous Oxygen Transfer Rates*

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Summary. Oxygen transfer has been studied in the *Pseudomonas ovalis* fermentation that quantitatively converts glucose into gluconic acid. The rate of oxygen uptake by this system was correlated with aeration rates and agitator speeds over wide ranges of these variables, by the introduction of a liquid expansion term. The technique described provides a biological method for studying oxygen transfer in the *P. ovalis* system; its general principles can be applied to other fermentations.

Introduction

The oxygenating capacity of fermentors has been evaluated by sulphite oxidation and polarographic methods.[1–5, 7, 9, 10, 12, 14–19, 21] Both techniques are only partially successful for this purpose; the first ignores the presence of micro-organisms,[1–5, 7, 10, 12, 15–19] the second requires artificial conditions such as removal of oxygen or poisoning of the cells.[1, 9, 14, 21]

This paper presents a new biological technique for the continuous and instantaneous measurement of oxygen transfer in systems containing microbial cells. The technique is based upon discovering a metabolic product that has a constant and quantitative relation to the oxygen consumed and which can be conveniently determined. Once this has been done, the rate of oxygen transfer is measured by observing the rate of formation of the selected product.

* Presented before the 136th National Meeting of the American Chemical Society, Atlantic City, N.J., September 1959.

The method will be illustrated by its application in the *Pseudomonas ovalis* fermentation that quantitatively converts glucose into gluconic acid and from which a correlation between oxygen uptake, power input, air flow, and liquid expansion were developed.

Development of the Empirical Equations

In submerged, aerobic fermentation systems, oxygen transfer is controlled by its movement from the air bubbles to the fluid, by diffusion in the fluid, or by transfer across the liquid–cell boundary. In our gluconic acid fermentation broths, the second step is assumed to be sufficiently rapid so that it does not become rate limiting. Oxygen entering the microbial cells is used in biochemical reactions; when these reactions are not limiting, the overall rate of oxygen utilization by the fermentation system is controlled either by the rate of oxygen transfer across the gas–liquid interface or across the liquid–cell boundary.

A transfer equation may be written in the following form:

$$\frac{dc}{dt} = KA\Delta f \tag{1}$$

When this equation is applied to a gas–liquid interface, oxygen transfer can be expressed as:

$$\frac{dc}{dt} = KA(f_g - f_l) \tag{2}$$

If the rate limiting step in an overall process occurs at the gas–liquid interface, then f_l will be negligible compared with f_g and equation (2) can be approximated by equation (3) or equation (3a).

$$\frac{dc}{dt} = KAf_g \tag{3}$$

$$\frac{dc}{dt} = KAy_g \tag{3a}$$

With ordinary air, where the partial pressure of oxygen is substantially constant, the oxygen transfer rate is approximately

proportional to the product of a transfer coefficient and the transferring area at the gas–liquid interface, i.e.

$$\frac{dc}{dt} \propto KA \tag{4}$$

Similarly, equations (5, 6, 6a, and 7) can be written for those systems where the overall oxygen transfer rate is controlled by the resistance at the liquid–cell boundary. In this case, it is assumed that f_s is negligible and that x_l is essentially constant and approximately equal to the oxygen solubility in the liquid.

$$\frac{dc}{dt} = K'A'(f_l - f_s) \tag{5}$$

$$\frac{dc}{dt} = K'A'f_l \tag{6}$$

$$\frac{dc}{dt} = K'A'x_l \tag{6a}$$

$$\frac{dc}{dt} \propto K'A' \tag{7}$$

In submerged fermentation systems, both the gas–liquid and liquid–cell boundaries are involved in oxygen transfer. For either boundary, if KA is considered to be a function of the factors in equation (8),

$$KA = F(P, G, \rho, \mu, d, L, \sigma, l) \tag{8}$$

A relation can be obtained by aid of dimensional analysis:

$$KA = k_1\rho G\left(\frac{L}{d}\right)^{k_2}\left(\frac{G\rho d^2}{\mu}\right)^{k_3}\left(\frac{Pd}{G\sigma}\right)^{k_4}\left(\frac{\sigma}{d^3G^2\rho}\right)^{k_5}\left(\frac{1}{L}\right)^{k_6} \tag{9}$$

In the present experiments, conditions were arranged so that the terms, L, d, μ, ρ, and σ, were constant in a given series of runs. Equation (9) therefore reduced to

$$KA = k_7P^{k_8}G^{k_9}E^{k_{10}} \tag{10}$$

where

$$E = \frac{1}{L} \times 100 \tag{11}$$

When the agitator speed was included in place of power input, as shown in equation (12),

$$KA = F(N, G, \rho, \mu, d, L, \sigma, l) \tag{12}$$

then a similar equation was derived which utilized N in place of P. This yielded:

$$KA = k_{11}N^{k_{12}}G^{k_{13}}E^{k_{14}} \tag{13}$$

The results obtained were correlated with equations (10) and (13). The coefficients and the exponential constants were determined by graphical trial and error analyses and the method of least squares. It should be noted that the derived equations are empirical in nature.

Materials and Methods

Pseudomonas ovalis NRRL–B–8 oxidizes glucose into gluconic acid as a terminal metabolic product. The reaction can be expressed in the following form:

$$C_6H_{12}O_6 + \tfrac{1}{2}O_2 \longrightarrow CH_2OH(CHOH)_4COOH$$

Lockwood, Tabenkin and Ward[11] reported that glucose present in the fresh medium was either converted into gluconic acid by the organism or it remained as unused glucose. This was confirmed by analysis of fermented media during the present study: results from 24-h culture media showed that 99·0 per cent of the glucose fermented was converted into gluconic acid.

A continuous recording technique was introduced by Finn[6] and developed by Gillies,[8] and West[20] to measure the instantaneous rate of acid formation in the lactic acid fermentation. The same technique was used in the present work to measure the rate of gluconic acid formation by resting cells of *Pseudomonas ovalis* NRRL–B–8. The rate of oxygen transfer into the fermenting broth was expressed in terms of the rate of acid formation.

(*a*) *Fermentor.* A 5-l. cylindrical glass fermentor was used in the present oxygen transfer study. This fermentor was 6 in. in diameter and 12 in. high. Four stainless-steel baffle plates, each $11{\cdot}5 \times 0{\cdot}625$ in., were fixed to the head plate. They were tied together by a stainless-steel ring at the bottom. The baffle

plates were placed at right angles with their surfaces perpendicular to the fermentor walls and their lower ends 0·5 in. above the bottom of the vessel. Through the head plate an agitator shaft was centrally mounted, on which an impeller was fixed at 2·5 in. above the bottom of the fermentor. The impeller was made of four $0{\cdot}625 \times 0{\cdot}625$ in. stainless-steel blades, set 90° apart with their flat surfaces perpendicular to the bottom of the vessel. An air pipe passed through the head plate; at its tip, a single hole sparger was attached with the aperture facing upward, 0·6 in. below the impeller.

(*b*) *pH Controlling System.* The calomel and glass electrodes were attached to the baffle plates of the fermentor so that their tips dipped into the broths. The electrodes were connected to a Bristol pH-controller. During an experiment, the gluconic acid produced by the *Pseudomonas ovalis* cells lowered the pH of the liquid. Once the pH reached a desired value, the controller started a pump that added a sodium hydroxide solution to the mash. The rate at which sodium hydroxide was added was proportional to the rate of gluconic acid formation. A diagram of the equipment used is shown in Fig. 1.

The sodium hydroxide solution was contained in a one-litre graduate, t, which was connected to the fermentor, m, through the pump, p. A float, s, rested on the surface of the sodium hydroxide solution. This float was fabricated from a glass bulb fastened to a light wooden stick to which an ink-pointer, w, was attached. The tip of the pen pressed against the surface of the drum, r, which was driven by a clock motor, u, at a speed of 24 or 12 rev/h, as desired. During an experiment, the ink-pointer traced a curve on a sheet of paper fastened to the surface of the drum. The vertical coordinate indicated the volume of the sodium hydroxide solution in the reservoir, t; the horizontal coordinate indicated the time elapsed. The rate at which sodium hydroxide was pumped into the fermentor at any instant was obtained by graphical differentiation of the curve using a platen that was specially designed for this purpose.

(*c*) *Accessories.* Air was obtained from the laboratory compressed air line; the air flow rate was measured with a rotometer. A stroboscope or a tachometer was used to measure the agitator speed. The level of the liquid in the fermentor was read against

a scale marked on the outside of the glass vessel and power input to the system through the agitator was measured with a spring-type dynamometer and a stroboscope.

(*d*) *Preparation of Cultures and Resting Cells of* Pseudomonas ovalis. The culture of *Pseudomonas ovalis* NRRL–B–8 used in this study was obtained from the Northern Utilization Research

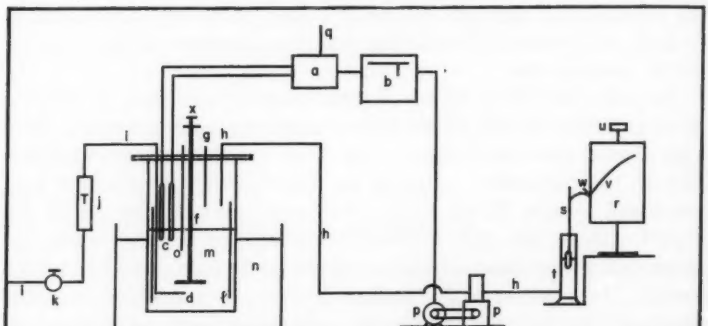

Fig. 1. Equipment used in oxygen transfer studies with *Pseudomonas ovalis*

a: pH meter
b: pH controller and recorder
c: Electrodes
d: Air sparger
e: Impeller
f: Agitator shaft
g: Sampling tube
h: NaOH feed line
i: Air line
j: Flow meter
k: Valve
l: Baffle plates
m: Fermentor
n: Constant temperature water bath
o: Thermometer holder
p: Sigma pump and motor
q: Power line
r: Kymograph drum
s: Float
t: NaOH solution reservoir
u: Clock motor
v: Curve drawn
w: Ink-pointer
x: Spring dynamometer

and Development Division of the United States Department of Agriculture at Peoria, Illinois. The medium used for culturing this organism was of the following composition:

glucose	50 g
KH_2PO_4	0·6 g
$MgSO_4$	0·25 g
urea	2·0 g
yeast extract	5·0 g
distilled water	1·0 l.

When a solid medium was required, 20 g of agar was also added.

In preparation for an experiment, a slant culture was first

inoculated and incubated for 24 h at 30°C. These cells were washed into one litre of fresh liquid medium in a 1·5-l. Florence flask. The liquid media used in the culture tubes and in the 1·5-l. flasks were of the composition given above.

The inoculated medium in the 1·5-l. flask was next incubated at 30°C on a rotary shaker that rotated the flask at 200 rev/min in a circle of one inch diameter. After 24 h, the cells were centrifuged from the broth and resuspended in 2 l. of a buffered glucose solution of the following composition:

glucose	50 g
KH_2PO_4	1·2 g
distilled water	2·0 l.

No nitrogen was present in the solution. This resting cell suspension was used for the respiration study carried out in the 5-l. fermentor.

(*e*) *Experimental Procedure.* Two series of experiments were conducted with resting cells of *Pseudomonas ovalis*; in the first, the combined effect of power input, agitation and air flow on the rate of oxygen transfer was studied; in the second, the effect of cell concentration on the rate of gluconic acid production was investigated.

In the first series of experiments, 880 ml of a 24-h *Pseudomonas ovalis* culture were centrifuged and resuspended to make approximately 2 l. of a cell suspension as previously described. The number of cells in the suspension was adjusted turbidemetrically to contain $1{\cdot}76 \times 10^{10}$ cells per ml.

The cell suspension was poured into the 5-l. fermentor, four drops of an antifoaming agent, Dow polyglycol P–2000, were added, the headplate was mounted, the agitator and air flow were started, and, finally, the pH controller was set at a value of 5·5. The changing level of the alkali solution in the graduate was automatically recorded on the drum, r, shown in Fig. 1. This drum turned once in 24 h.

In each run, the acid production rate started at a low value but increased quickly until after 30 to 60 min a steady rate of acid production was established. This was indicated by a constant slope of the trace drawn on the drum, r. The experiment was then considered to be under way. It was continued for one to two hours. This time was needed to allow the line drawn on the drum to

become sufficiently extended for its slope to be accurately measured.

One level of agitator speed and one air flow rate were studied at different agitator speeds and air flow rates using the methods described. The results obtained are given in Table I. Equa-

Table I. Effects of power input, agitation and air flow on the rate of gluconic acid production by *Pseudomonas ovalis* NRRL–B–8

Agitator speed, N rev/min	Air flow rate, G vvm	Power input, P h.p./l. h $\times 10^{-3}$	Rate of acid production, mole/l. h $\times 10^{-3}$	Liquid volume expansion E, per cent by vol
470	1·16	10·7	3·75	9·3
370	1·16	7·4	3·75	4·9
470	0·31	12·3	4·0	5·8
470	1·81	10·5	4·0	9·5
470	0·73	11·8	4·75	4·9
100	1·16	2·2	2·0	1·5
270	1·16	4·4	3·5	2·6
370	1·16	7·4	4·24	3·5
270	1·81	4·3	3·75	3·0
270	0·31	4·6	3·0	2·5
100	1·81	2·2	1·75	1·5
100	0·73	2·2	1·62	1·3
270	1·81	4·4	3·0	4·8
270	0·73	4·5	3·12	2·4

tion (10), developed by dimensional analysis, suggested that a linear correlation should result when the proper groups of variables were plotted on logarithmic coordinates, so this procedure was followed.

A graphical trial and error method was used to determine the exponential constants; the values of k_8, k_9, k_{10}, thus determined, were 0·8, 0·2 and $-0{\cdot}5$, respectively. This is shown in equation (14):

$$KA = 0{\cdot}2P^{0{\cdot}8}G^{0{\cdot}2}E^{-0{\cdot}5} \text{ moles } O_2/\text{l. h} \qquad (14)$$

The coefficients were determined by the method of least squares. The overall correlation showing the effect of power input on the system is graphically presented in Fig. 2, while that for equation (14) is given in Fig. 3.

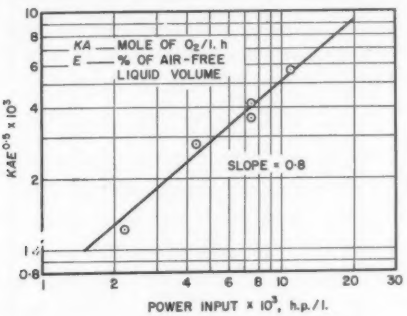

Fig. 2. Effect of power input on the rate of oxygen transfer in the *Pseudomonas ovalis* system

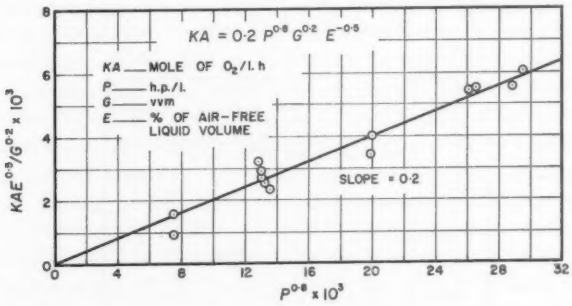

Fig. 3. Effects of power input and air flow on the rate of oxygen transfer in the *Pseudomonas ovalis* system

When the factor N was substituted for power input, equation (15) was obtained:

$$KA = 3{\cdot}43 \times 10^{-6} N^{1{\cdot}2} G^{0{\cdot}2} E^{-0{\cdot}5} \text{ moles } O_2/\text{l. h} \qquad (15)$$

The corresponding plot is shown in Fig. 4.

The effect of cell concentration on the rate of oxygen transfer was also investigated by the experimental methods previously described, using two levels of agitator speed as well as two air flow rates. The results of the experiments are shown in Fig. 5, where it is seen that a linear relation exists between the cell concentration and the rate of acid production.

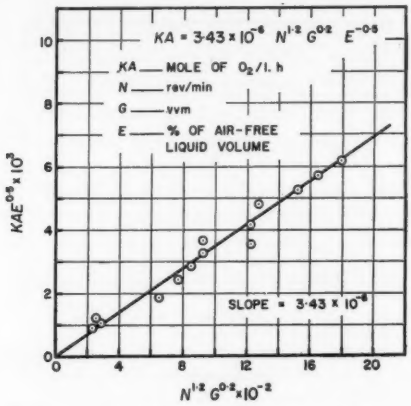

Fig. 4. Effects of agitation and air flow on the rate of oxygen transfer in the *Pseudomonas ovalis* system

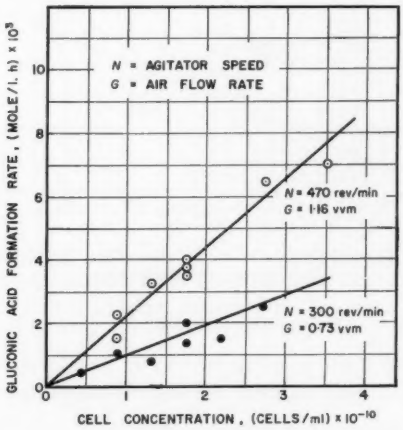

Fig. 5. Effect of cell concentration on the rate of gluconic acid formation in the *Pseudomonas ovalis* system

Discussion

The *Pseudomonas ovalis* fermentation of glucose to gluconic acid proposed in this paper is a biological method for determining

the rate of oxygen uptake. The method is based upon the assumption that a definite ratio exists between the amount of oxygen consumed and of gluconic acid produced. Because microbial cells were actually involved in this oxygen uptake measurement, the method provides a biological way of evaluating the oxygen transfer performance of fermentors.

In actual fermentations, three phases are involved: gas, liquid and solid. In such systems, the overall oxygen transfer rate may be controlled by either the gas–liquid or the liquid–cell interfaces. In either case, the rate of oxygen uptake is proportional to the product of a transfer coefficient and a transferring area, as shown in equations (4) and (7). When the gas–liquid interface is limiting, the transfer coefficient and the transferring area are those associated with the gas–liquid interface; in the other cases, they refer to the transfer coefficient and the transferring area at the liquid–cell boundary. If the overall oxygen uptake rate is limited by the liquid–cell boundary, and if the bacterial cells involved are of similar size and shape, the rate of oxygen uptake, as shown in equation (7), should be proportional to the cell concentration of the system. This linear relationship is observed in Fig. 5. The derivation of equation (7) was based upon two assumptions; namely, that f_s in equation (5) is negligible and that f_l is approximately constant and close to the oxygen transfer potential of a broth saturated with oxygen. These assumptions might profitably be considered in connection with the runs for which data are presented in the table.

At present, there is no adequate method for measuring f_s, the oxygen transfer potential of the bacterial cells. Probably oxygen is instantaneously fixed and quickly used by the enzyme systems; hence, the resistance to further oxygen transfer as well as the value of f_s should be negligible.

The second condition, namely that of a high and essentially constant f_l, probably existed in these runs. This presumption proceeds from the following interpretation of the results.

First of all, if the liquid–cell interface is controlling, the f_l will almost certainly be high.

Secondly, using sulphite oxidation data taken in the same fermentor with the same agitator speeds and air flows, the oxygen supply rate was calculated to be in the range of 0·01 moles/l. h.

Based upon data in the table, the oxygen requirements for glucose oxidation were approximately one-tenth of this.

Such a difference between the rate of oxygen supply and demand should have produced a high f_l value.

Finally, the data in Fig. 5 show a five-fold rise in the oxygen uptake rate caused by increasing the cell concentration in the suspension; a further increase could be expected from extrapolation of the curves.

This would suggest that eventually, as the cell concentration was increased, the correspondingly higher oxygen demands could cause the gas–liquid interface to become controlling. However, in our runs, which were conducted with low cell concentrations, the effect of resistance at the gas–liquid interface must have been small. Since both the agitator speed and the air flow were constant in these runs, overcoming a resistance at the gas–liquid interface could only have resulted from an increase in the potential difference, as shown by equation (2). Because f_g is constant, f_l is the only term that could have varied; so, in order to produce a five-fold increase in the oxygen transfer across the gas–liquid interface, the value of f_l in the experiments reported in the table would have needed to be at least four-fifths of the saturation level at low cell concentrations.

Based upon the same reasoning, the variability in f_l must have been confined to a maximum of one-fifth of the saturation value when cell concentration was low.

These considerations suggest that a high and relatively constant dissolved oxygen concentration existed in the broth during runs discussed in the table; they also help to substantiate a contention that the observed effects of agitation and power changes on oxygen uptake rates resulted from the alteration of transfer resistances at the liquid–cell interface.

From preliminary experiments, it was found that the rate at which gluconic acid was formed was not materially affected by temperature changes. The effects of physical factors such as air flow rate, power input, and agitator speed on the rate of acid production were shown in equations (14) and (15). From these data it was apparent that in these instances, oxygen transfer was controlled by the physical process of mass transfer. This follows because chemical reactions are affected by temperature changes

but do not show appreciable response to variation of physical factors such as agitation.

From other preliminary experiments, it was found that changes in the concentrations of glucose, phosphate and gluconic acid in the liquid had no effect upon the rate of acid formation. This indicates that oxygen transfer was the rate controlling factor.

These considerations, together with the linear relationship found between cell concentration and oxygen transfer rates, indicate that the bacterial surface area can be directly associated with the rate controlling mechanism in the oxygen transfer process of the *Pseudomonas* fermentation and suggest that, in these studies, the transferring step across the liquid–cell interface did control the overall utilization of oxygen.

Acknowledgment. The financial support of this work through a grant from the National Science Foundation is gratefully acknowledged.

Nomenclature

A	Transferring area
A'	Transferring area where liquid–cell-wall interface is rate controlling
d	Impeller diameter
E	Liquid volume expansion factor defined by equation (11)
f_g	Oxygen transfer potential of gas phase
f_l	Oxygen transfer potential of liquid phase
f_s	Oxygen transfer potential of solid phase
G	Air flow rate
k_1, k_2, k_3, etc.	Constants
K	Transfer coefficient
K'	Transfer coefficient where liquid–cell-wall interface is rate controlling
l	Total liquid volume increase due to air held-up
L	Total volume of the air-free liquid
N	Agitator speed
P	Power input per unit volume of liquid
x_l	Mass fraction of oxygen in liquid phase
y_g	Mass fraction of oxygen in gas phase

σ	Surface tension of the liquid
ρ	Density of the liquid
μ	Viscosity of the liquid
$\frac{dc}{dt}$	Overall rate of oxygen uptake
Δf	Oxygen transfer potential difference

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Handling of Antifoam Oils for Fermentations*

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Summary. Control of foams by oil additions is of large economic importance to the fermentation industry. Excessive foaming causes loss of material and contamination, while excessive oil additions may decrease the product formation. Antifoam oils may be synthetic, such as silicones or polyglycols, or natural, such as lard oil or soybean oil. Either will substantially change the physical structure of foam, principally by reducing surface elasticity.

Industrial antifoam systems usually operate automatically from level-sensing devices. Methods for metering of oil under aseptic conditions are: timed delivery through a solenoid, two solenoids with an expansion chamber between, a motor-driven hypodermic syringe, and certain industrial pumps.

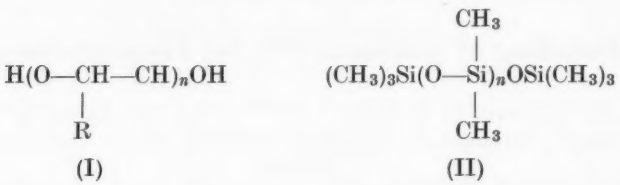
Distribution devices can improve efficiency over straight-pipe entry. Types described are spray distribution, diverter bars, and wick devices.

Severe foaming in a fermentation vessel may cause contamination or loss of material, either of which will decrease yields and invalidate experimental data. While there are reports of satisfactory foam control by mechanical means, the great majority of troublesome foams are treated with defoaming agents. In addition to foam control, oils added to fermentations usually participate in the nutrition of the culture and may sometimes be added in excess for crude pH adjustment with the acids produced by metabolism of the oil.

Lard oil, soybean oil, linseed oil, tall oil, and rape-seed oil are examples of natural antifoam agents. Some alcohols are suitable for antifoam use; those derived from fatty acids seem best, but octyl alcohol is sometimes used. Polyglycol (I) or silicone (II)

* Presented before the 136th National Meeting of the American Chemical Society, Atlantic City, N.J., September 1959.

oils with the general formulae shown control most foams very well and are not metabolized appreciably.



(I) (II)

With some fermentations, a small amount of these agents in the make-up controls foam throughout the run. More commonly, however, these oils inhibit antibiotic production and must be avoided or restricted. The inhibition may result from the very small air bubble size observed with these agents.

Antifoam agents have profound effects on the fermentation environment. In almost all cases, a large excess is detrimental to product biosynthesis. For some fermentations, the maximum foaming propensity is at the start of the run. Evidently the surface active agents are modified by metabolism because the onset of good cultural growth reduces the tendency to foam. To hold the contents in the tank without using inhibitory amounts of antifoam agent, the air flow and agitation must be kept below desirable levels and the environment is not optimum.

The effect of oils on oxygen transfer has been well documented, but there are several other gross environmental effects of oil. When oil is added to foam, the bubbles collapse rapidly, and the dissolved gases and the agitation pattern are greatly changed.

Analysis of the exit gas shows that oxygen uptake and carbon dioxide production drop markedly soon after an application of antifoam. This results from decreasing effective contact between the culture and air trapped in foam. The foam bubbles must contain stale air because of the immediate release of carbon dioxide as the foam is broken. The effect on aeration and agitation is shown by visual inspection of the liquid and by the mixing power requirements. In a glass fermentor, the air bubble size may be observed to change markedly because of the effect of antifoam on surface properties. Thus gas transfer and the aeration of the culture are changed by shots of oil. Fig. 1 shows a wattmeter

record of mixing power. Mixing power drops gradually because of a build up of air entrainment which lowers the liquid density and partially floods the impellers. Defoaming gives a sharp increase in power because of de-gassing. Approximately 20 per cent more power is required to stir large fermentors just after a shot of oil. With these environmental changes influenced by antifoam oils, a detailed study of their nature, application, and engineering problems seems justified.

Kitchener and Cooper[1] have reported on foams in terms of stabilizers, foam-breakers, and foam inhibitors. The primary

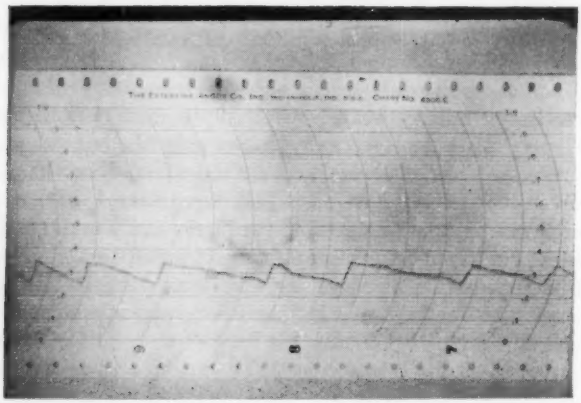


Fig. 1. Wattmeter record of mixing power

requirement for foaming is 'film elasticity' so that the bubbles can respond to stresses and changes. A foam starts as spherical, wet bubbles, which drain to give a network of films defining polyhedral bubbles. The curvature of the walls indicates that unequal pressures exist in different bubbles.

Film elasticity and damping of shock-waves are essential to give mechanical strength to foam, while double-layer repulsions and steric interactions provide the forces to preserve a foam. The presence of a solid phase permits discontinuities by adsorption; this affects the surface properties and may favour foaming. Flow by surface transport is required to get stabilizing agents

evenly distributed and to prevent local concentration as some bubbles collapse. The prime action of a defoamer is its ability to reduce surface elasticity. To do this, a foam inhibitor must displace the stabilizer, and low solubility aids in maintaining high surface concentration under dynamic conditions. Most antifoam oils fall into this category. Direct foam breakers such as ether and alcohols act by local reduction of surface tension as they penetrate the film; this allows neighbouring bubbles to rip the weakened film apart. This foam breaking action may cease when the system is saturated with the organic solvent because surface tension discontinuities cannot be created in adjacent bubbles.

Some compounds promote drainage by lowering viscosity and thus accelerate the life-cycle of the foam. All foams must collapse to reach equilibrium because the decrease in surface energy yields a lower energy state. Electrolytes can help dissipate double-layer repulsion forces by improving the electrical conductance of the film. Other aspects of industrial foams have been reviewed in more detail by Gaden and Kevorkian.[2]

Turning now to the engineering problems of foam, a prime requirement is to avoid contaminating the fermentation. Antifoam oil has been regarded as requiring 'dry heat' sterilization conditions, that is 160°C for 2 to 3 h. Another opinion is that 127° for 90 min is adequate.[3] We sterilize large batches of oil in tanks with direct steam for 2 to 3 h at 127°, but small flasks of oil for pilot-plant fermentor feeds are sterile after about 20 min in the autoclave. Natural oils tend to decompose to form carbonaceous or greasy sediments which may clog control valves or meters. Solenoid valves with perpendicular stems have oil around the plunger in such a way that deposits can lodge in the crevices and jam motion, but in-line valves are flushed clean by flow. Storage vessels and pipe lines for oil are usually kept warm to reduce viscosity and to aid flow.

Materials of construction for natural oils must resist organic acids formed by degradation, and diaphragms must not be softened by the bad combination of oil and moisture at sterilizing temperatures. Stainless steel lines and Teflon gaskets and diaphragms are used in our oil systems although other metals and some new grades of synthetic rubbers are satisfactory.

For detection of foam in the fermentor, several methods have been reported. Manual control by inspection and valve turning is poor because close attention is too wasteful of man power, and errors, as mentioned previously, readily lead to serious loss of contents and to contamination.

Automatic oil addition depends upon reliable sensing of the foam level in the vessel. Although some mechanical device such as a paddle or a float could sense the foam level, commercial installations use electrical methods because no sterility problems are created. A conducting probe is insulated from the tank wall and placed so that the maximum allowable rise of foam completes the circuit and the current flow from the probe through the liquid to the tank wall is detected. Relays, often in electronic tube circuits, are actuated and can thus control the valves or pumps in the oil system. Some sort of delay is often included to prevent oil shots from a splash or transient foam rise. An installation has been reported which uses a searching switch to scan the electrodes on all the fermentors and actuates an alarm to call the operator for antifoam additions.[4] Another reliable device is adjusted to sense the change in capacitance as foam becomes the dielectric in the danger zone in the tank. Again simple relay circuitry is used to signal the antifoam oil controls. We have had experience with both conductance and capacitance probes and have no strong preference which would justify changing from our conductance probe type sensing devices. The conductance probes are sometimes shorted by a collection of materials across the insulation at the point of entry into the tank, and the result is addition of excess antifoam oil in amounts which may spoil the batch. The capacitance detector eliminates this problem because it is a passive element that cannot be fouled. Occasional tube failures are to be expected in electronic relay circuits, and there is no such thing as a fail-safe antifoam system because too much oil or too much foam is ruinous. In practice, the detection systems cause much less trouble than do the solenoid valves on the oil lines.

The metering of sterile oil is a rather specialized operation. A wide variety of methods have been tried in our laboratories. Drip or sight feeders are of little concern to commercial fermentations where manual control is seldom used except in emergencies.

For large fermentors, timed delivery is sometimes adequate to obtain proper shot size. The foam detection apparatus trips a timer which in turn opens a valve for the desired interval. With fermentations which metabolize the oils with beneficial effects, this crude control may be desirable because of the tendency to exceed the minimum amounts of oil required to break the foam. Fig. 2 shows a temperature chart from our Tippecanoe plant.

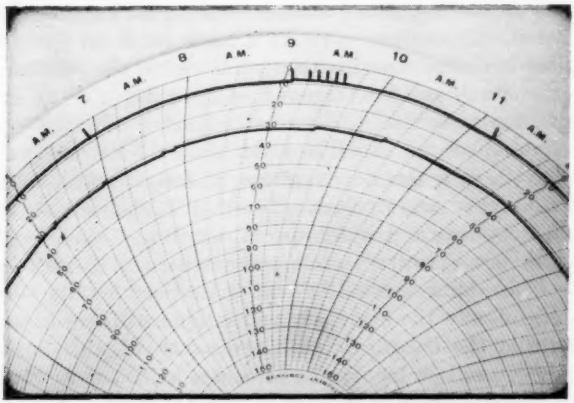


Fig. 2. Antifoam record on temperature chart

There they have installed an inexpensive device to record antifoam shots on the temperature records. A sequential relay sends a signal after every six shots to a recording pen which marks a convenient location on the temperature chart.

Fig. 3 shows a modification of a drum feeder that was placed inside the fermentor. Unfortunately, during sterilization it trapped moisture which collected under the oil and floated it out when not needed. For closed system work where contamination is a problem, feeders of this sort present problems which outweigh their possible advantages. Fig. 4 shows a system used by Stefaniak and co-workers[5] which used the blow-pot principle to give an exact shot of oil by forcing the contents of a small chamber into the fermentor with air pressure. The solenoid which equalizes pressure to allow the chamber to fill is shut

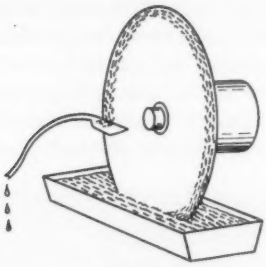


Fig. 3. Disc feeder

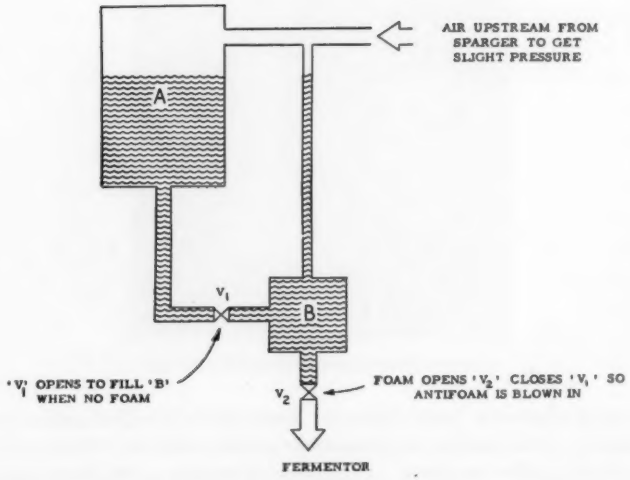


Fig. 4. Blow-pot antifoam system

off while the other solenoids open to allow sterile air to move the oil.

The antifoam installation on our 30-l. fermentors uses a pressurized oil system with a needle valve to add the oil in drops. In the case of laboratory or small pilot-plant fermentors, on–off

delivery of oil is poor because normal oil shots must range from one or two drops to at most two or three millilitres.

Fig. 5 shows a far better system in use on our more modern 67-l. fermentors. The oil is metered by sequential timing of solenoid valves with an expansion chamber. When a shot is called for, the inlet valve opens so that pressurized oil fills the

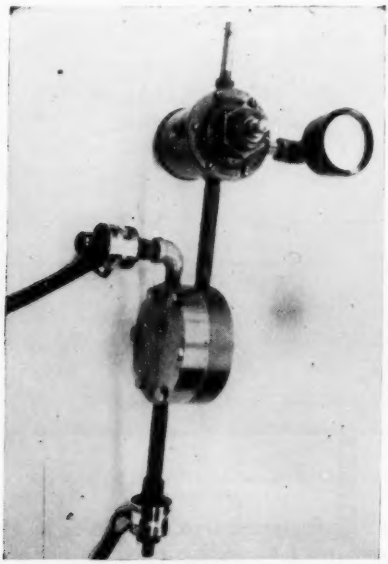


Fig. 5. Diaphragm expansion chamber

metering chamber, next the inlet closes and the discharge opens to permit the chamber air pressure to deliver the oil. Both valves are closed between shots. A source of trouble with this system is valve leaks or sticking which may allow full flow through the system when the other valve is actuated. The calibrated oil reservoir is used to obtain accurate usage data for development and scale-up purposes. Fig. 6 shows a system developed in our pilot plant using a motor-driven hypodermic syringe. One minor drawback is low capacity which necessitates frequent refilling. The unit is moderately expensive if purchased,[6] but can be

fabricated in the company shops. Shots are added dropwise slowly to give fine control of foam; this method is highly recommended for small fermentors.

Several problems must be solved before conventional metering pumps can be used to add antifoam oils. First of all, expense all but eliminates the pumps which are capable of doing the job.

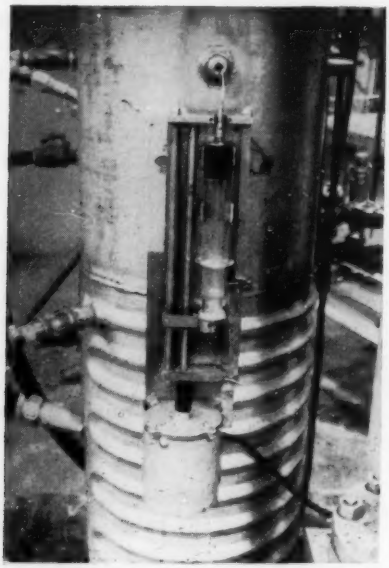


Fig. 6. Motor-driven hypodermic syringe

The other considerations are sterility and absolute shut-off. Many people in the industry are cautious in accepting pumps in which the fluid is retained by packing or seals. Even if phenol greases are used, there is a chance that some error or scratch or worn part will permit entry of a foreign organism. Eventually in all plants, contamination of sensitive fermentations occurs, and all possible sources are investigated and eliminated. We have had oil meters with several years of satisfactory service on penicillin and streptomycin, but they were considered dangerous, and were removed for the sensitive erythromycin fermentation.

Gear pumps, reciprocating piston pumps, centrifugal pumps, and all such devices using a gland or seal through which the moving elements are driven, are possible contamination hazards.

The absolute shut-off feature is difficult to obtain because a fairly good sized pump is required to get the shot in rapidly, thus the sealing area is fairly large. Furthermore, maintenance is possible only between fermentation runs, and wear can cause failure or leaks at inopportune times. Since there is often a long interval between oil shots, even small leaks are intolerable to oil-sensitive fermentations such as streptomycin. Many metering pumps use check valves; these are subject to leaks especially in service on a combination of steam, water and oil. This is not meant as a criticism of the pumps, which are fine for continuous service, but points out the problems involved in intermittent service.

We have evaluated metering pumps on our 12,000-gal production fermentors. Plunger pumps operate well on a contamination resistant fermentation like penicillin, but might be dangerous in other cases. Diaphragm check-valve pumps have no sterility hazard and work reliably, but are quite expensive. We found flexible liner pumps to be satisfactory except for infrequent liner failures and a tendency to leak if the pump stopped in the wrong position. The leakage was corrected by using a down-stream pressure relief check valve.

Peristaltic pumps which squeeze tubing are good for special projects, but deterioration of the tubing during sterilization and from contact with the oil make them unsuitable for routine use. A clamp described by Anderson and co-workers resembles a peristaltic pump and should have the same limitations.[7] A diagram of a small pump of our own design is shown in Fig. 7. The placing of a check valve on the plunger permits operation within an enclosed line with no sterility problems. Plunger motion is controlled from a solenoid, energized intermittently from a timer.

The manner of entry of oil into the fermentor is also important. Continuous oil addition on a schedule is often a good way to control foam. We have found that total oil consumption can be one-fourth less than with strictly demand additions. With suitable metering, the foam can be held at a roughly constant

level, providing a more constant environment. The drawback of the method is the inability to cope with unexpected heavy foaming.

Spray application has been frequently mentioned in the literature.[8] Our experience with sprays is that any advantage over the direct stream is too slight to justify the maintenance of a sprayer. Total oil consumption is greater with a spray; perhaps some oil is lost by entrainment in the exhaust air.

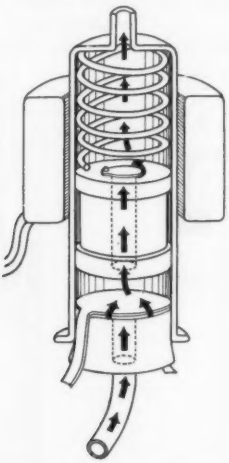


Fig. 7. Solenoid pump

Nelson, Maxon and Elferdink[9] have reported a system which roughly approximates the distribution of a spray by adding the antifoam to a divertor fastened to the impeller shaft. This inexpensive feature cannot be undesirable and may be of some benefit.

We feel that we have developed a superior distribution technique in our laboratories, as shown in Fig. 8. Wicking material is supported on pipes or wire mesh and soaked with oil as required. The foam level rides just below the wicks, and oil is drawn into the foam in small amounts. This minimizes excess oil being consumed by metabolism. We have observed oil usage reductions

of from 25 to 50 per cent with wicks or with carefully adjusted continuous feeds. Furthermore, a constant foam level minimizes the severe environmental changes mentioned earlier.

The hair-pin shaped wick was observed to plug with sediments and congealed oil after varying lengths of service. Steam could be used to free the passages, but a better solution seems to be the tent-shaped wick support, shown in Fig. 9. This allows oil to be dripped on, rather than to be forced outward through holes in

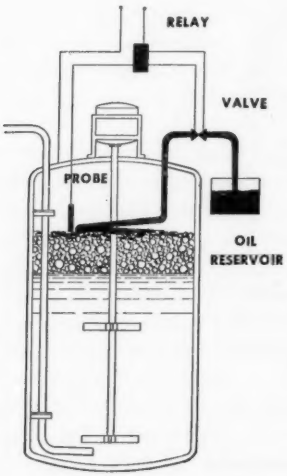


Fig. 8. Antifoam tubular wick

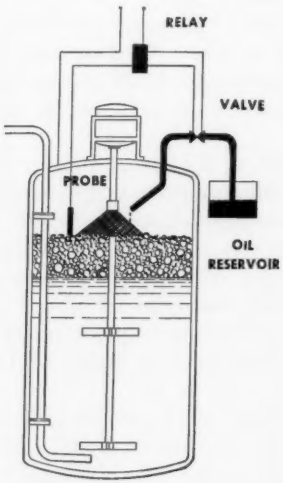


Fig. 9. Tent-shaped wick

the supporting pipe, and there is nothing to clog. The wick method has also been useful in controlling foam in our filtration surge tanks where flow removes oil rapidly if added to the liquid. Further processing was easier when the troublesome excess oil was eliminated.

Future research with foams and defoaming agents should lead to better mechanical breakers and to oils with better and more specific properties. It is likely that oils will continue to be fed to fermentations, and adequate, modern methods are available.

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Electronic Computer Solution for the MPN Equation used in the Determination of Bacterial Populations

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Summary. The utility and the practical precision of the Most Probable Number method for determining bacterial populations is currently restricted by a lack of adequate tables of solved examples or alternatively by an easy and quick solution to individual problems. Where an electronic computer is available, these restrictions need not apply. A computer programme and example solution for tabulated data and individual problems are presented in this paper.

Introduction

The extinction dilution technique is one of two standard methods for the determination of the viable population of bacteria in a sample of material.[3] As has been pointed out,[1] this method developed from the Phelps Index, through McCrady's calculations, to the generalized relations of Halvorson and Ziegler.[1] These latter, rather elegant equations, have been solved for only a few combinations of numbers, which in addition have been limited to decimal dilutions and small numbers of tubes in each dilution.

Halvorson and Ziegler[2] pointed out that deviation from the mode is high using only five tubes in each of three decimal dilutions, but narrows rapidly with increased numbers of tubes in each dilution. Unfortunately, practical application of the Most Probable Number (MPN) method is severely restricted by the few tabulated solutions of the general equations that are available. Actually, only the tables for five tubes[3] and for ten tubes in decimal dilutions are readily accessible,[1] although solutions for a

few other miscellaneous combinations can be obtained.[3] Since this is only a restriction of equation solutions and not an inherent defect of the method, increased value of the MPN technique should result from the availability of other solutions. This paper presents an electronic computer programme capable of solving any reasonable combination of like numbers of tubes in decimal or other manner of dilutions.

Theory

Let several dilutions be used and several tubes be inoculated with each dilution of a bacterial culture. Halvorson and Ziegler's notations and equations are used, where:

X = number of bacteria per ml in the median significant dilution,
n_1 = number of samples of volume a_1 ml taken,
p_1 = number of failures out of n_1 trials, where failure means growth in the tubes,
q_1 = the number of successes out of n_1 trials, where success means no growth in the tubes.

If the set of tubes are inoculated with a_1 ml in each of the n_1 tubes in set 1, a_2 ml in the n_2 tubes of set 2 and so on; and if p_1 of the tubes of set 1 show growth, p_2 of set 2, . . . where a_i, n_i and p_i refer to the ith dilution, then a generalized form of Halvorson and Ziegler's equation gives:

$$\sum_{i}^{N} a_i\left(\frac{p_i}{1-e^{-a_i X}} - n_i\right) = 0 \qquad (1)$$

In addition, the percentage probability that this growth pattern will occur for the computed value of X is obtained from:

$$\log_e P = \sum_{i}^{N}\left[\log_e\left(\frac{n_i!}{p_i!(n_i-p_i)!}\right) - a_i(n_i-p_i)X + a_i p_i \log_e(1-e^{-a_i X})\right] \qquad (2)$$

Since the statistical equation (1) is implicit in the desired unknown, X, it is most easily solved by an iterative, successive

approximation method. In this case the Newton–Raphson method was used. Here a new approximation $\bar{X}$ to the unknown X is generated with each iterative cycle from

$$\bar{X} = X - \frac{G(X)}{dG/dX} \tag{3}$$

until $\bar{X} = X$ is established within a previously allowable error. Specifically, for this problem

$$G(X) = \sum_{i}^{N} a_i \left(\frac{p_i}{1 - e^{-a_i X}} - n_i \right)$$

and

$$\frac{dG}{dX} = -\sum_{i}^{N} \frac{a_i^2 p_i e^{-a_i X}}{(1 - e^{-a_i X})^2}$$

Using the above equations, the computer flow diagram shown in Fig. 1 was developed.

A programme to fit a computer was then produced from this flow sheet. The actual machine programme used depends upon the computer available; we used an *IBM–650 equipped with index accumulator and floating point accessories.

Results and Discussion

The programme described above can be used to solve quickly individual problems or to prepare tables of solutions. For example, a complete table containing all possible combinations of ten tubes in decimal dilutions was solved for X and P in 1 h 20 min of computer time. A condensed version of this table is given by Halvorson and Ziegler.[1] The machine solutions were identical with those given in the above article except for one or two numbers. For example, at code (10, 2, 0) the machine solution is $X = 0{\cdot}329$ and $P = 12{\cdot}53$ per cent, the published data show $X = 0{\cdot}329$ and $P = 18{\cdot}14$. In this case, if the middle dilution, which is the median significant dilution, were 0·1 ml then x, the number of bacteria per ml in the original undiluted sample, would be 0·329 multiplied by the reciprocal of the dilution or 3·29 bacteria per ml.

* The detailed programme can be furnished upon request to the authors.

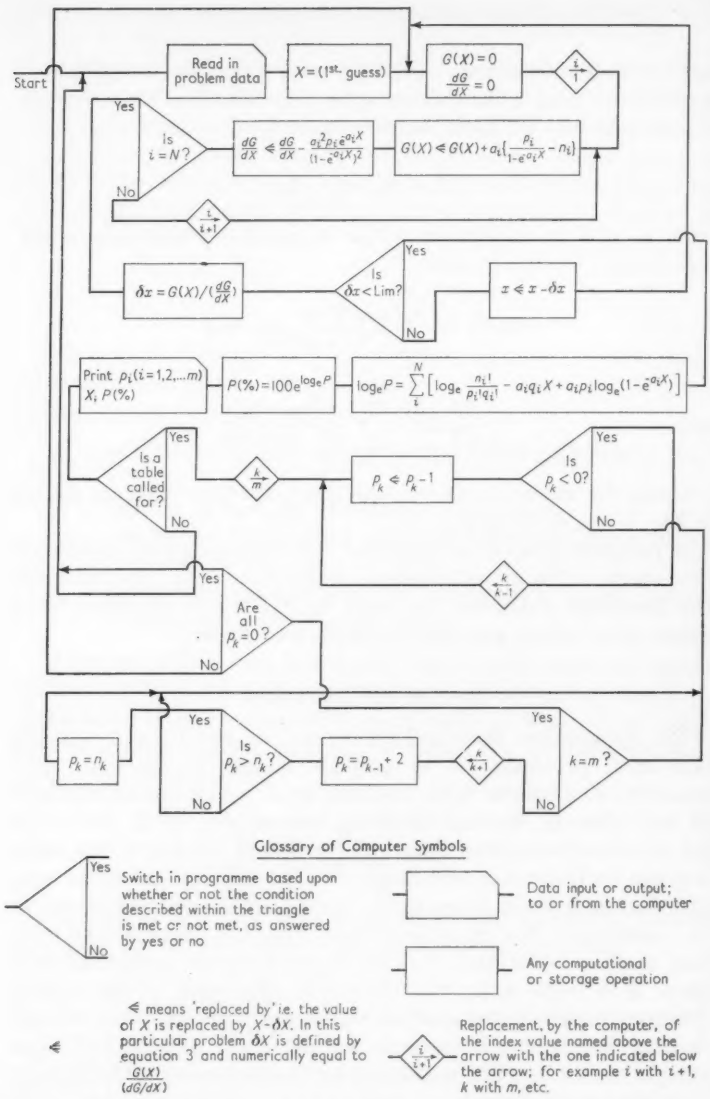
Start
Read in problem data
X = (1st. guess)
$G(X)=0$
$\frac{dG}{dX}=0$
$\frac{i}{1}$
Yes
Is $i=N$?
No
$\frac{dG}{dX} \Leftarrow \frac{dG}{dX} - \frac{a_i^2 p_i e^{a_i X}}{(1-e^{a_i X})^2}$
$G(X) \Leftarrow G(X) + a_i\{\frac{p_i}{1-e^{a_i X}} - n_i\}$
$\frac{i}{i+1}$
$\delta x = G(X)/(\frac{dG}{dX})$
Is $\delta x <$ Lim?
Yes
No
$x \Leftarrow x - \delta x$
Print $p_i (i=1,2,\ldots m)$ X, $P(\%)$
$P(\%) = 100 e^{\log_e P}$
$\log_e P = \sum_i^N \left[\log_e \frac{n_i!}{p_i! q_i!} - a_i q_i X + a_i p_i \log_e (1-e^{-a_i X})\right]$
Is a table called for?
$\frac{k}{m}$
$P_k \Leftarrow P_k - 1$
Is $P_k < 0$?
$\frac{k}{k-1}$
Are all $P_k = 0$?
$P_k = n_k$
Is $P_k > n_k$?
$P_{k-1} + 2$
$\frac{k}{k+1}$
$k=m$?
Glossary of Computer Symbols
Switch in programme based upon whether or not the condition described within the triangle is met or not met, as answered by yes or no
Data input or output; to or from the computer
Any computational or storage operation
$\Leftarrow$ means 'replaced by' i.e. the value of X is replaced by $X-\delta X$. In this particular problem δX is defined by equation 3 and numerically equal to $\frac{G(X)}{(dG/dX)}$
Replacement, by the computer, of the index value named above the arrow with the one indicated below the arrow; for example i with $i+1$, k with m, etc.

Fig. 1. Computer programme for solution of equations for determination of the Most Probable Number of bacteria in a sample and of probabilities for occurrence of the indicated codes of bacterial growth in the culture tubes

Results having a P greater than 0·0049 for eight tubes in each of three decimal dilutions are given in Table I. As shown by the calculations of Halvorson and Ziegler,[2] this selection of tubes and dilutions provides a considerable improvement in the anticipated precision for the calculated value of X with a minimum of additional laboratory effort. Specifically, the deviations shown by Halvorson and Ziegler[2] for 97 per cent of the data indicate an improvement for eight tubes as compared with five tubes per dilution as follows: with eight tubes the deviations above and below the mode are 155 and 63 per cent respectively, while with five tubes the values are 260 and 70 per cent. Obviously an even larger number of tubes improves the precision further; but at ten tubes the increase is small, the values being 130 per cent above and 58 per cent below the mode respectively. Hence, routine determinations of the most probable number of bacteria by the dilution method would materially benefit from the use of eight tubes in each of three dilutions rather than the customary five tubes. For this reason a table of solutions for the most probable number of bacteria present in the median significant dilution, using eight tubes in each of three decimal dilutions, is included in this paper.

Table I. The most probable numbers of bacteria per ml of the median dilution of sample using eight tubes in each of the decimal dilutions

Code	X	P	Code	X	P
8 8 7	20·8	39·3	8 6 6	2·84	0·02
8 8 6	13·9	31·1	8 6 5	2·50	0·16
8 8 5	9·82	28·2	8 6 4	2·18	0·75
8 8 4	7·02	27·1	8 6 3	1·89	2·66
8 8 3	5·10	26·5	8 6 2	1·63	6·92
8 8 2	3·85	23·9	8 6 1	1·38	12·22
8 8 1	3·01	16·9	8 6 0	1·15	11·39
8 8 0	2·40	6·85	8 5 6	2·13	0·00
8 7 8	5·96	0·00	8 5 5	1·89	0·04
8 7 7	5·08	0·04	8 5 4	1·66	0·26
8 7 6	4·33	0·21	8 5 3	1·44	1·27
8 7 5	3·69	0·87	8 5 2	1·23	4·45
8 7 4	3·14	2·67	8 5 1	1·030	10·66
8 7 3	2·67	6·36	8 5 0	0·842	13·61
8 7 2	2·26	11·40	8 4 5	1·48	0·01
8 7 1	1·91	14·10	8 4 4	1·30	0·10
8 7 0	1·59	9·26	8 4 3	1·11	0·62

Table I. *Continued*

Code	*X*	*P*	Code	*X*	*P*
8 4 2	0·940	2·88	7 0 0	0·155	8·17
8 4 1	0·774	9·22	6 6 1	0·308	0·00
8 4 0	0·622	16·01	6 6 0	0·277	0·02
8 3 5	1·18	0·00	6 5 1	0·273	0·03
8 3 4	1·02	0·04	6 5 0	0·244	0·13
8 3 3	0·867	0·30	6 4 2	0·269	0·01
8 3 2	0·718	1·80	6 4 1	0·241	0·12
8 3 1	0·582	7·70	6 4 0	0·214	0·63
8 3 0	0·467	17·98	6 3 2	0·238	0·03
8 2 4	0·807	0·01	6 3 1	0·211	0·37
8 2 3	0·672	0·13	6 3 0	0·186	2·29
8 2 2	0·550	1·03	6 2 2	0·209	0·06
8 2 1	0·445	5·81	6 2 1	0·184	0·84
8 2 0	0·362	17·73	6 2 0	0·160	6·08
8 1 3	0·522	0·05	6 1 2	0·182	0·08
8 1 2	0·427	0·47	6 1 1	0·158	1·29
8 1 1	0·350	3·44	6 1 0	0·135	10·95
8 1 0	0·287	13·34	6 0 2	0·156	0·05
8 0 2	0·338	0·13	6 0 1	0·134	1·03
8 0 1	0·280	1·17	6 0 0	0·113	10·40
8 0 0	0·231	5·67	5 5 1	0·207	0·01
7 7 1	0·547	0·01	5 5 0	0·185	0·04
7 7 0	0·484	0·02	5 4 1	0·184	0·03
7 6 2	0·530	0·01	5 4 0	0·163	0·23
7 6 1	0·471	0·05	5 3 2	0·182	0·01
7 6 0	0·415	0·15	5 3 1	0·161	0·14
7 5 2	0·458	0·03	5 3 0	0·141	1·13
7 5 1	0·404	0·20	5 2 2	0·160	0·02
7 5 0	0·355	0·64	5 2 1	0·140	0·42
7 4 3	0·446	0·01	5 2 0	0·121	4·04
7 4 2	0·395	0·07	5 1 2	0·139	0·04
7 4 1	0·347	0·55	5 1 1	0·120	0·87
7 4 0	0·304	2·09	5 1 0	0·101	9·83
7 3 3	0·386	0·01	5 0 2	0·119	0·04
7 3 2	0·340	0·13	5 0 1	0·101	0·94
7 3 1	0·298	1·16	5 0 0	0·083	12·75
7 3 0	0·259	5·15	4 4 0	0·128	0·09
7 2 3	0·333	0·01	4 3 1	0·127	0·05
7 2 2	0·292	0·18	4 3 0	0·110	0·57
7 2 1	0·255	1·83	4 2 1	0·109	0·22
7 2 0	0·220	9·53	4 2 0	0·093	2·67
7 1 3	0·287	0·01	4 1 2	0·108	0·02
7 1 2	0·251	0·16	4 1 1	0·092	0·59
7 1 1	0·217	1·97	4 1 0	0·076	8·70
7 1 0	0·186	12·12	4 0 2	0·091	0·02
7 0 2	0·214	0·08	4 0 1	0·075	0·84
7 0 1	0·183	1·11	4 0 0	0·060	15·52

Table I. *Continued*

Code			X	P	Code			X	P
3	4	0	0·101	0·03	2	1	0	0·039	6·35
3	3	1	0·100	0·02	2	0	2	0·051	0·01
3	3	0	0·085	0·28	2	0	1	0·038	0·62
3	2	1	0·085	0·11	2	0	0	0·026	24·54
3	2	0	0·070	1·72	1	3	0	0·049	0·05
3	1	2	0·084	0·01	1	2	1	0·049	0·02
3	1	1	0·070	0·38	1	2	0	0·036	0·53
3	1	0	0·056	7·56	1	1	1	0·036	0·12
3	0	2	0·069	0·02	1	1	0	0·024	5·00
3	0	1	0·055	0·74	1	0	2	0·036	0·01
3	0	0	0·041	19·12	1	0	1	0·024	0·50
2	4	0	0·079	0·01	1	0	0	0·012	35·14
2	3	1	0·079	0·01	0	2	0	0·023	0·19
2	3	0	0·066	0·12	0	1	1	0·023	0·04
2	2	1	0·065	0·05	0	1	0	0·011	3·33
2	2	0	0·052	1·03	0	0	1	0·011	0·33
2	1	1	0·052	0·23					

To use this table for determining bacterial populations, Halvorson's[1] notations apply. In each dilution, the code refers to the number of tubes showing growth out of eight tubes inoculated. For example, if eight tubes of suitable liquid media are inoculated with 1 ml, eight with 0·1 ml and eight with 0·01 ml of sample and growth occurs in seven, five, and one tube out of each set upon incubation, then 0·404 bacteria were present per ml of the median dilution or 4·04 bacteria per ml of undiluted sample. The probability of this combination of tubes developing is 0·20 per cent from an infinite number of trials using a suspension containing 4·04 bacteria per ml. It should be noted that $X = x$ when the median significant dilution is 1, i.e. not diluted; for other median significant dilutions, x equals X divided by the fractional value of this dilution. In the above instance (0·404) (1/0·1) = 4·04 bacteria per ml of the original sample

The equations, and therefore the machine solutions, are not restricted to decimal dilutions or to any prescribed number of tubes per dilution. For example, a table could be developed using 15 tubes per dilution, five such dilutions and a two-fold ratio between sets of tubes; or if enough similar problems were available requiring solution to warrant the use of a computer, they could be readily and quickly solved.

References

[1] Halvorson, H. O. and Ziegler, N. R. *J. Bact.*, **25**, 101–121 (1933)
[2] Halvorson, H. O. and Ziegler, N. R. *J. Bact.*, **26**, 559–567 (1933)
[3] Standard Methods for the Examination of Water, Sewage and Industrial Wastes, 10th ed., American Public Health Association

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Rheological Examination of some Fermentation Broths*

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Summary. The broths of four important industrial fermentations, namely nystatin, penicillin, and streptomycin fermentations, and a fermentation used for steroid hydroxylation, were examined rheologically. Considerable variation in rheological properties was demonstrated among these fermentations as well as throughout the course of the nystatin and streptomycin fermentations. These latter fermentations, employing *Streptomyces* strains, were mostly Newtonian in behaviour. Departures from this behaviour twice in the streptomycin fermentation resulted in plastic characteristics. The other two fermentations employing moulds were found to be markedly non-Newtonian, either plastic or pseudo-plastic in their behaviour.

Rheological properties of most fermentation broths change appreciably during the course of fermentation. These properties influence the nature of the fluid regime in fermentors and, as a consequence, are important factors when considering mass and heat transfer. Therefore, a knowledge of the rheological behaviour of fermentation broths is an important prerequisite for an intelligent understanding of these transport processes. This paper briefly reviews some of the mathematical flow models used to characterize fluids and reports the rheological examination and analysis of several industrially important fermentation broths containing filamentous micro-organisms.

Flow Models and Previous Studies

The laminar behaviour of any fluid is defined by the relationship between shearing stress, τ, and velocity gradient or shear rate, dv/dy. All fluids which exhibit direct proportionality between shear stress and shear rate as expressed in equation (1) are classed as Newtonian fluids.

$$\tau = \mu(dv/dy) \tag{1}$$

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This is the simplest relationship possible and only one property, μ, the viscosity, characterizes the laminar flow behaviour of the fluid.

All other fluids are classed as non-Newtonian. Two non-Newtonian flow models commonly assumed in chemical engineering practice[7] are described by equations (2) and (3).

$$\tau - \tau_y = \eta(dv/dy) \tag{2}$$

$$\tau = K(dv/dy)^n \tag{3}$$

Fluids defined by equation (2) are called plastic fluids. These materials have a definite yield stress, τ_y, which must be exceeded before flow will begin. Once the fluid flows the shear stress is proportional to the velocity gradient. The proportionality constant, η, is called rigidity to distinguish it from viscosity. Equation (3), an empirical power-law, defines any fluid which exhibits an approximately straight line when shear stress is plotted against velocity gradient on logarithmic coordinates. It has been found most useful for fluids termed pseudo-plastic as well as certain plastic fluids. A pseudo-plastic fluid does not exhibit a linear relationship between shear stress and velocity gradient when plotted on arithmetic coordinates. K is called the consistency index and n is called the flow behaviour index. It will be noted that if n is equal to one, equation (3) represents Newtonian behaviour and K equals the viscosity. For plastic or pseudo-plastic fluids n is less than one.

Because of their interlacing mycelial networks or long flexible unidimensional cell chains, mould, actinomycetic, algal, and certain bacterial cultures should impart some degree of structural rigidity to their broths. Rheological properties reported for streptomycin broth,[5] for water suspensions of an unidentified mould,[1] and for penicillin broth,[2] indicate that these fluids approximate to plastic behaviour. Little is known about the quantitative relationship between rheological properties and cellular concentration in these fluids. The literature pertaining to the rheological properties of fermentation broths has been reviewed elsewhere.[3]

Rheological properties may be used to demonstrate growth in fermentation broths. The rigidities obtained during the course

of a penicillin fermentation[2] indicate that small changes in cellular concentration appear as large changes in rigidity. Often, too, fermentations are carried out in media containing various suspended solids which obscure accurate cell weight determinations. In these situations a rheological property is particularly useful as a measure of growth. Torque at a prescribed cup speed measured in a MacMichael viscosimeter has been used[4] to show the growth of *Streptomyces garyphalus* in a medium containing soybean meal, glucose and calcium carbonate, as used in the production of cycloserine.

Experimental Procedures

In some recent studies, rheological data were obtained from several industrial fermentations which utilized filamentous micro-organisms. The fermentations studied, the micro-organisms used, and the nature of the nutrient in which the micro-organisms were grown are listed in Table I.

Table I. Characteristics of fermentations examined

Process	Micro-organism	Principle nutrients in media	
		Energy source	Nitrogen source
Nystatin	*Streptomyces noursei*	glucose	soybean meal
Penicillin	*Penicillin chrysogenum*	lactose	cornsteep liquor
Steroid hydroxylation	*Coniothyrium hellbori*	glucose	inorganic salts
Streptomycin	*Streptomyces griseus*	glucose	soybean meal

A four-speed Brookfield Syncro-lectric viscosimeter with its spindle guard removed was used in these studies. A series of specially constructed cylindrical spindles, $\frac{1}{4}$ in., $\frac{1}{2}$ in. and $\frac{3}{4}$ in. in diameter by $2\frac{9}{16}$ in. in length were used according to the single bob technique of Krieger and Maron[6] to obtain the data required to calculate and construct shear diagrams of the various broth samples. The broth was placed in a 200-ml beaker (4 in. high with an inside diameter of 2 in.) which was large enough for the spindle to rotate in an effectively infinite medium, unaffected by wall proximity. Fig. 1 shows the viscosimeter and spindles employed.

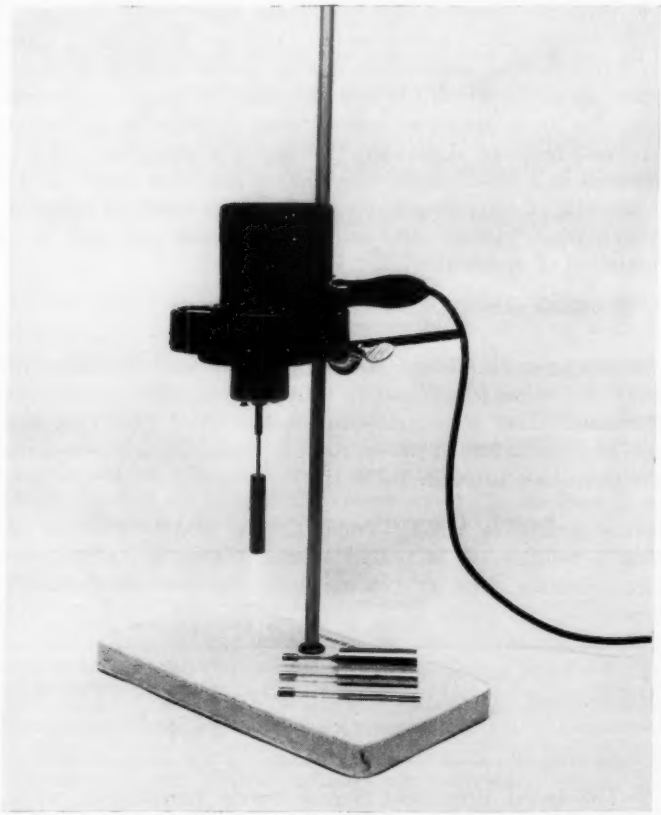

Fig. 1. Brookfield Syncro-lectric viscosimeter and spindles

Equations (4) and (5) are adapted from equations developed by Krieger and Maron. They permit the calculation of shear stress and velocity gradients from direct measurements made using the Brookfield viscosimeter.

$$\tau = C\theta/2\pi h r_b^2, \tag{4}$$

$$dv/dy = 4\pi\theta(dN/d\theta) \tag{5}$$

Table II contains typical viscosimeter data obtained for a 43-h old *Coniothyrium hellebori* broth. Fig. 2, a graphical representation of these data, is used to obtain values of $dN/d\theta$ needed in equation (5) to calculate velocity gradients. Shear stress is

Table II. Data for 43-h *C. hellebori* Broth

Brookfield data		Graphically determined data. $dN/d\theta$, rev/min (deflection unit) (slope, Fig. 2)	Calculated data	
Spindle speed N, rev/min (fixed)	Bob deflection θ, deflection units (observed)		Shear stress g cm^{-1} sec^{-2}	Velocity gradient, dv/dy (eq. (5))
0·1	17·5	0·014	3·17	3·08
0·2	22·5	0·022	4·08	6·10
0·5	31·5	0·050	5·70	19·80
1·0	37·8	0·100	6·84	47·50

obtained directly from the deflection at each spindle speed using equation (4). A logarithmic plot of shear stress and velocity

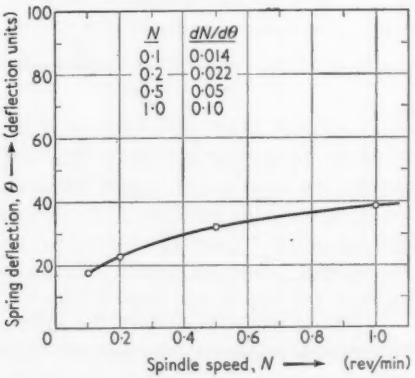

Fig. 2. Graphical representation of observed viscosimeter data for the determination of $dN/d\theta$

gradient, as shown in Fig. 3, yields a consistency index of 2·3 pseudo-poises * and a flow behaviour index of 0·29 for this sample.

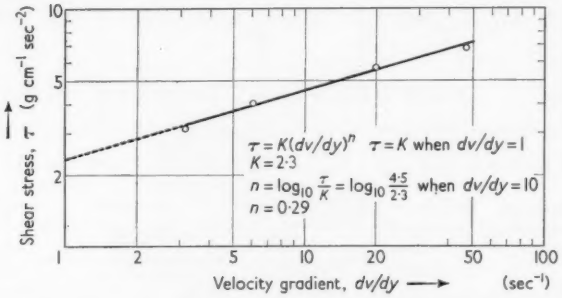

Fig. 3. Logarithmic plot of the shear diagram for the calculation of flow behaviour index, n, and consistency index, K

Results and Discussion

Shear diagrams of broths at various ages from each of the fermentations listed in Table I are illustrated in Figs. 4–7. The non-Newtonian nature of some of these broth samples is clearly

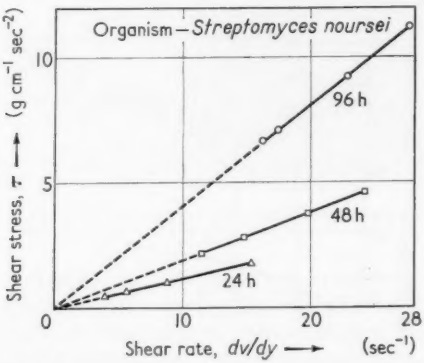

Fig. 4. Shear diagram of a nystatin broth at age 24, 48 and 96 h

* A pseudo-poise is here defined as a unit of measure for the consistency index, which varies in its dimensions with the value n, the dimensionless flow behaviour index. The units of the pseudo-poise are (gram)/(centimetre) (second)$^{2+n}$.

evident at certain periods during the fermentation. Despite the mycelial character of the antinomycete *Streptomyces noursei*, nystatin broth displays a remarkably good Newtonian behaviour. The curves in Fig. 4 represent samples with viscosities ranging between 11·5 cP at 24 h to 40 cP at 96 h.

The penicillin broth samples represented in Fig. 5 are more pseudo-plastic than plastic in their behaviour, contrary to an earlier report.[2] The two samples shown in Fig. 5 had consistency indices of 82 and 51 pseudo-poises and flow behaviour indices of

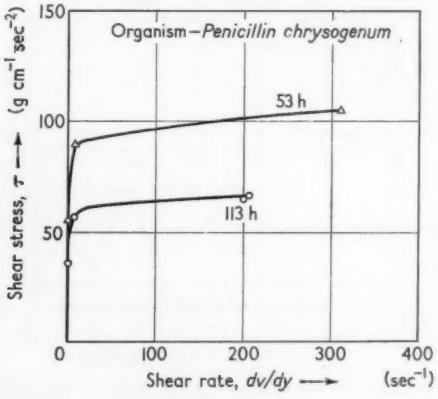

Fig. 5. Shear diagram of a penicillin broth at 53 and 113 h

0·040 and 0·048, at ages 53 and 113 h, respectively. If measurements at low rates of shear are not taken, these samples might easily be mistaken for plastic fluids with very high yield stresses.

In Fig. 6, samples of broth containing *C. hellibori* showed both plastic and pseudo-plastic behaviour. From age 19 h to age 94 h, the yield stress of this broth increased almost eight-fold from 0·3 g cm^{-1} sec^{-2} to 2·3 g cm^{-1} sec^{-2}, and the rigidity of the broth increased almost eight-fold from 4 cP to 31 cP.

Shear diagrams of streptomycin broth samples, constructed in Fig. 7, indicated plastic behaviour at 24 h and Newtonian behaviour at 48 and 96 h, with a rigidity of 52 cP and viscosities of 18 and 38 cP, respectively. The plastic behaviour of the broth

is in agreement with earlier observations,[5] but the Newtonian behaviour at other times is surprising.

Figs. 4–7 indicated that a wide variation in fluid behaviour occurs not only among fermentations, but even during the course

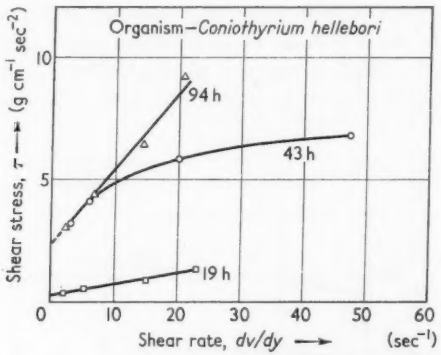

Fig. 6. Shear diagram of a steroid hydroxylation broth at 19, 43 and 94 h

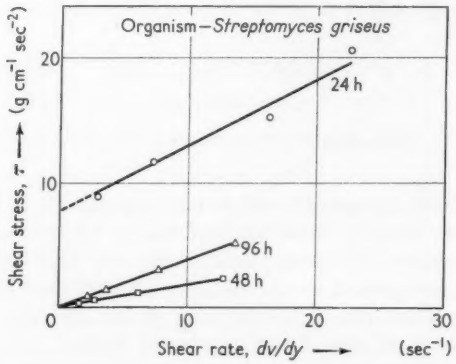

Fig. 7. Shear diagram of a streptomycin broth at 24, 48 and 96 h

of the same fermentation. This point was examined in greater detail during the course of the nystatin and streptomycin fermentations.

Fig. 8 illustrates the variation in the consistency and flow behaviour indices during the nystatin fermentation. The broth behaved as a Newtonian fluid until after it reached a maximum viscosity of 48 cP at age 36 h. This is indicated by a constant flow behaviour index of 1·0. After this the broth varied but slightly from Newtonian behaviour. The two visocity peaks at 12 and 36 h were characteristic of this fermentation. They probably indicate a rise and fall in the complexity of the mycelial networks. A metabolic explanation is at present not at hand.

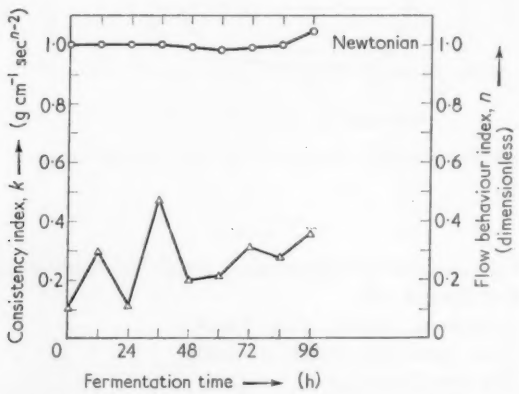

Fig. 8. Consistency and flow behaviour indices for a nystatin fermentation

The variation in the consistency index and the flow behaviour index (or viscosity) throughout the streptomycin fermentation is illustrated in Fig. 9. Again, two pronounced peaks, this time representing marked plastic behaviour, were observed. One occurs early during the fermentation and is due to an advanced mycelial network development during a highly aerobic phase of growth. This is followed by abrupt fragmentation. Later in the fermentation another period of more complicated development occurs. Rheological properties of the broth are very sensitive indicators of such changes in mycelial character.

These data demonstrate that the physiological age of the fermentation can effect the rheological behaviour of fermentation broths.

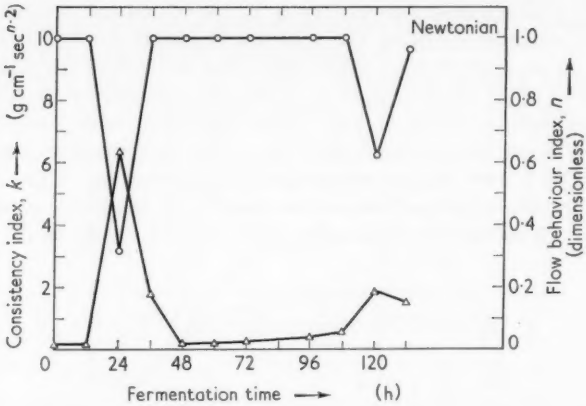

Fig. 9. Consistency and flow behaviour indices for a streptomycin fermentation

Notation

C	Viscosimeter spring constant, g cm^2 sec^{-2} (deflection unit)$^{-1}$
h	Bob height, cm
K	Consistency index, g cm^{-1} sec^{n-2}
n	Flow behaviour index, no units
N	Bob rotational speed, rev/min
r_b	Bob radius, cm
dv/dy	Shear rate, sec^{-1}
η	Rigidity, g cm^{-1} sec^{-1}
θ	Bob deflection, deflection units
π	Constant, 3·14
μ	Viscosity g cm^{-1} sec^{-1}
τ	Shear stress, g cm^{-1} sec^{-2}
τ_y	Yield stress, g cm^{-1} sec^{-2}

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Surface Culture in the Mass Growth of Bacteria*

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Summary. The present study was conducted to determine the feasibility of growing bacteria in quantity on solid media in closed vessels. Five techniques were investigated in the laboratory, including growth on agar, growth on packing in beds, growth on solids in a fluidized bed, growth in foamed medium, and growth on semipermeable membranes. Data obtained from the laboratory experiments were used to scale up these techniques on paper and compare the projected plants with a conventional submerged fermentation production. All the techniques compared unfavourably with submerged culture on the basis of plant and operating costs, operating complexity, equipment complexity, and safety. One technique of surface culture, growth on semipermeable membranes, appears to offer a decided advantage over submerged culture, in that the cell product can be recovered directly as a paste. Further work on this technique is recommended.

Introduction

In 1958, this laboratory concluded a feasibility study of mass culture of bacteria on solid media. The ultimate objective was the development of a practical method for growing microorganisms in closed vessels characterized by large surface-to-volume ratios.

Before discussing the laboratory results, a few comments about mass culture are in order. At present, most large-scale fermentations are conducted to obtain some product of cell metabolism. Usually, cell growth is of interest only as it affects product yield. In such cases, submerged culture techniques have proved far more economical than surface techniques and great effort has been spent in adapting surface fermentations to submerged culture.

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When the desired product of culture is the cell mass, however, the culture requirements may change. Surface culture offers the possibility that cells may be harvested in the form of a heavy paste rather than as a dilute suspension. Surface culture also offers the possibility that the air requirements of aerobic organisms can be met more readily and that cell metabolism will be entirely aerobic. Depending upon the desired characteristics of the microbial cells, surface culture would appear to offer some theoretical advantages. Realization of the potential advantages, of course, depends upon the development of a practical method of surface culture.

Experimental Results

Inoculation

The efficiency of surface inoculation appears to be important in any surface culture scheme. As a matter of convenience, we studied the problems of inoculation on agar plates.

Table I summarizes data obtained with agar plates. As shown in the table, the inoculum size did not affect the yield

Table I. The effect of inoculum size on the total recovery of *S. marcescens* from agar plates*

Inoculum size, organisms per plate	Number of replicate tests	Bacteria recovered per plate, organisms $\times 10^9$
20×10^9	8	$128 \pm 21{\cdot}0$
1000	3	94 ± 74
800	5	109 ± 31
600	4	128 ± 29

* Medium: tryptone glucose extract agar (20 ml/plate)
Incubation: 24 h at 37°C

significantly. Plates that were inoculated with dilute slurries gave rise to discrete colonies in which growth was elevated from the agar surface; plates that were inoculated with concentrated slurries gave rise to thin films of bacterial growth that covered the entire plate surface. It was concluded that growth was

limited by the nutrient supply and that complete utilization of the surface is not necessary for maximum yield.

The conclusion that nutrients were limiting in the surface culture of *Serratia marcescens* led to a study of the effect of medium thickness on bacterial growth. Plates containing 5, 10, 15, or 20 ml of medium were streaked with inoculum so that approximately 1,000 organisms were applied to each plate. After 24 h of growth, the organisms were harvested and counted. The data obtained are summarized in Table II.

Table II. The effect of agar thickness on the growth of *S. marcescens* in Petri plates*

Agar volume, ml	Agar depth,† mm	Bacterial counts, billions of organisms	
		Total harvest	Count per ml of original medium
20	2·8	210	10·5
15	2·2	157	10·4
10	1·4	108	10·8
5	0·7	41	8·2

* Medium: tryptone glucose extract agar (Difco)
Incubation: 24 h at 37°C
† Calculated from the diameter of the plate (90 mm) assuming uniform distribution of the medium

As shown in this table, total recoveries were related to medium depth. It is interesting to note that the total recovery per unit of medium was independent of the medium depth except in the case of plates containing 5 ml of medium. These plates dried out during incubation and it is believed that dehydration is responsible for the reduced growth.

This study was repeated and fluid medium was studied in parallel with the solid medium. The data obtained are shown in Table III. As shown in this table, total yield from solid medium and from fluid medium increased as the medium depth increased. The yield per unit of medium was uniform in the case of the solid medium but decreased as the depth of fluid medium increased. On the basis of these data, it was concluded that oxygen was limiting in the case of fluid cultures and that nutrients were

Table III. The effect of medium thickness on the growth of *S. marcescens* in Petri dishes*

Medium volume, ml	Medium thickness, mm	Bacterial count, billions of organisms†			
		fluid medium		solid medium	
		per ml of medium	total‡	per ml of medium	total
6	0·84	3·0	18	10·7	64
8	1·12	1·8	14	10·8	87
10	1·4	2·8	28	10·4	104
12	1·68	2·1	25	9·8	118
14	1·96	1·6	22	8·2	116
16	2·24	1·4	22	9·9	159
18	2·52	1·5	27	12·9	230
20	2·8	1·6	32	9·9	199

* The medium was tryptone glucose extract broth or agar (Difco); incubation was 24 h at 37°C without aeration or agitation; inoculum was 1000 organisms per plate
† Average of three determinations
‡ Total count per Petri plate

limiting in the case of surface cultures. Surface, *per se*, appeared to have no special effect on bacterial growth. Complete utilization of medium surface appeared to be unimportant to maximum yield of bacterial cells.

Packed Bed Studies

The results obtained in studies of agar plates were supported by the data obtained when bacteria were grown on nutrient-bearing supports in packed beds. Fluid and solid media were used on a variety of supporting solids. In some cases, the medium was circulated over the supports; in some cases, medium was neither circulated nor renewed. These supports were inoculated by flooding the bed with inoculum, by spraying inoculum on supports in a rotating drum, or by submerging supports in inoculated medium before they were charged into the bed.

Bacteria were removed from the supports after a suitable incubation period by agitating the support media in wash fluid. This process produces dilute suspensions of bacteria that may be heavily contaminated with material from the inert supports.

Contamination is especially severe in the case of alundum, or alumina supports.

The method of inoculation had a marked effect on the distribution of bacteria within the growth system, as shown by the distribution of bacterial pigment. The total yield, however, was not affected significantly by the method of inoculation.

Table IV. Total numbers of *S. marcescens* recovered from different medium-bearing supports

Support	Size	Volume retained nutrients	Bacteria recovered ($\times 10^9$)	
			total	per ml original medium
Pelletized alumina	$\frac{1}{8}$-in.	200	980	4·9
Glass beads	6 mm	20	56	2·8
Berl saddles	$\frac{1}{4}$-in.	100	276	2·8
Quartz sand	1 mm	55	99	1·8

Table IV summarizes part of the data obtained in studies of coated media. The vessels used in the studies had a capacity of 500 ml and, assuming 25 per cent head space, could be expected to produce harvests of approximately $7{,}500 \times 10^9$ organisms at concentrations approximating to 20×10^9 per ml. As shown in this table, the greatest total yield was obtained when pelletized alumina was used as the inert medium support. The yield per ml was approximately half of that obtained on agar plates. On the basis of bacteria per unit of fermentor volume, the yield was approximately one-tenth of that obtained in submerged culture. Yields from other support media were even less favourable.

On the basis of the studies summarized in Table IV, it is concluded that the basic concept of nutrient-bearing solids in mass surface culture of bacteria is impractical. It would appear that nutrients will always be limiting in such a system and that the net effect of the support material is the reduction of nutrient medium volume. If a highly porous support structure were utilized, the reduction in nutrients might be overcome. The pelletized alumina approaches this condition. The problems of gas exchange are magnified in such a system, however, and the problems of bacteria recovery are greatly increased.

Fluidized Bed Studies

A brief study was made of the growth of *S. marcescens* on clay particles in a fluidized bed. Attapulgus clay was fluidized and was then moistened with nutrient medium to a moisture content of approximately 20 per cent. *S. marcescens* was introduced into the bed with the nutrient medium. During operation of the bed, the moisture level was maintained through the addition of de-ionized water. Samples of the clay were removed at hourly intervals for bacterial and moisture determinations.

S. marcescens did not survive in the bed under the conditions of operation. It is possible to select a solid phase that would permit higher bed moistures without agglomeration. The technique is limited by the considerations mentioned for packed beds of nutrient-bearing solids, and is considered impractical for mass culture.

Foamed Medium Studies

Systems in which bacteria were grown on foam were also investigated. Yields from three different systems were approximately 1/1000 of those expected from similar equipment used for submerged cultures. When the medium was foamed by dripping the inoculated medium onto a sintered glass air distributor plate, bacteria were actually killed. It was tentatively concluded that excessive aeration was responsible for the decrease in viable count, although this point was not pursued.

Foam generators have interesting possibilities as research tools in studies of microbial growth, but appear unsuited to the mass culture of micro-organisms.

Semipermeable Membrane Studies

The most promising departure from submerged culture techniques was a system in which *S. marcescens* was grown on the outer surface of a semipermeable tube. In the laboratory, sections of cellulose sausage casing containing 100 ml of medium, and presenting a superficial surface of 39 in^2 were inoculated with *S. marcescens*. The casing was then incubated under controlled conditions of temperature and humidity for 24 h. Growth was scraped from the surface as a paste.

In the initial study of the system, approximately 1·3 g of paste were recovered from each tube. This product contained $1{,}000 \times 10^9$ viable bacteria per gram. The yield per ml of nutrient medium was approximately 11×10^9 bacteria. This value approximates to that obtained in the studies made with agar plates.

Study of the system was continued, to establish the effect of nutrient concentration on microbial growth. The results of this study are summarized in Table V. As shown here, double

Table V. Data obtained during the growth of *S. marcescens* on semipermeable tubes containing media of different concentrations*

Medium strength	Weight of paste, g	Bacterial recoveries, organisms ($\times 10^9$)			
		total	per gram of product	per ml of original medium	per square inch of surface
Single					
a.	1·03	1000	971	10·0	33
b.	1·10	980	898	9·8	33
Double					
a.	1·52	1850	1233	18·5	56
b.	1·52	1770	1180	17·7	53
Triple					
a.	1·04	1350	1290	13·5	40
b.	1·37	1640	1190	16·4	50
Quadruple, quintuple, and sextuple	No perceptible growth				

* Incubation was 24 h at 90°F and 100 per cent relative humidity. Original medium volume was 100 ml.

strength medium yielded approximately twice the yield obtained with single strength medium. When the medium strength was tripled, a slight reduction in yield was obtained in comparison with that of double strength medium. Further increases in nutrient concentrations prevented microbial growth. Yields are related to nutrient supply rather than the growth area.

In general, it appeared that the growth of bacteria on semipermeable membranes is feasible. Bacterial recoveries from the experimental systems approximated to those of submerged

culture in terms of yield per unit of nutrient medium volume. No effort was made to establish optimum conditions for bacterial growth. Manipulation of the medium composition, selection of the membrane, regulation of the air flow and selection of the point of aeration offer opportunities for increasing the yields of bacteria that can be obtained through this technique of surface culture.

Engineering Analysis

Since the objective of the research programme was the establishment of the feasibility of surface culture techniques, projections were made from the laboratory studies to commercial-scale operations. These projections were made to aid in the engineering appraisal of the experimental culture techniques. All schemes were scaled directly from the laboratory equipment to produce $6{\cdot}6 \times 10^{16}$ organisms in a batch process. Cost estimates were made in accordance with conventional procedures. Equipment costs were adjusted to the level of February, 1958, by means of the Marshall and Stevens Index.

Table VI presents a summary of the estimated costs for five hypothetical schemes as compared with a conventional deep vat culture operation. As this table shows, submerged culture is superior to the other schemes in terms of equipment complexity, operational complexity, and installed fermentor cost. The medium, inoculum, and fermentor volumes for submerged culture are smaller than for the other schemes. The semipermeable membrane device yields the most concentrated product, by far, and requires essentially no external air supply. These factors may be very important on more detailed investigation.

It seems doubtful that surface culture studies will ever lead to the development of a technique suitable for all fermentation or growth problems. In those cases where the desired result of microbial growth is cell accumulation, however, surface culture techniques offer advantages over submerged culture ones. It also appears that the growth of organisms on semipermeable membranes might simplify the recovery of some fermentation products from beers through the isolation of the organisms from the fluid phase of the culture. Data obtained here and at other laboratories with this type of culture device point to the

Table VI. Summary of data on estimates of materials, equipment, and costs for conventional and hypothetical schemes

Basis: (1) One batch in 24-h day (assume plant operates 330 days per year)
(2) Product from each batch contains $6{\cdot}6 \times 10^{16}$ viable cells
(3) Analysis limited to fermentation step

	Conventional fermentor	Scheme 1—agar on plates in a tower (119 plates)	Scheme 2—agar pellets in a tumbling blender	Scheme 3—packed bed with porous alumina pellets	Scheme 4—medium foamed in a closed system	Scheme 5—growth on porous-membrane tubing
Medium volume, gal	855	706	706	3,500 (in the medium)	1,560 (in the medium)	1,200
Inoculum volume, gal	45	94	94			360
Product volume, gal	855	235	235	7,000	1,250	13
Fermentor volume, gal	1,500	8,240	1,900	6,700	11,200	1,800
Air-flow rate, ft^3/min	19	0·42	0·42	41	0	recirculating
Fermentor installed cost, dollars	6,880	272,500	50,000	83,000	100,000	14,000
Raw materials cost, dollars	471	476	476	1,370	544	740*
Depreciation cost per batch, dollars	2	82	15	25	30	4
Operating complexity rating	good	poor	poor	poor	fair	poor
Equipment complexity rating	good	poor	poor	poor	fair	poor
Safety rating	good	poor	poor	fair	fair	poor
Product contamination rating	good	poor	poor	poor	fair	good

* Includes $200 for cost of tubing

usefulness of the technique for some purposes. The true measure of the value of this culture concept will be obtained only when the system is scaled-up physically and the potential benefits are great enough to warrant further study of the system.

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The Removal of Nitrogen and Phosphorus from Sewage Effluents through the Use of Algal Culture*†

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Summary. This study is designed to establish fundamental data which will permit the eventual application of algal culture to the removal of nutrients from sewage plant effluents. A test cell is described which gives reproducible data when environmental parameters are varied. The effect of changes in temperature, light intensity and culture density as reflected in the growth rate, are shown, and the influence of these parameters on the removal of nitrogen and phosphorus under the test conditions indicated. The importance of an adequate carbon source and proper pH control is demonstrated. The organisms used were *Scenedesmus* and *Chlorella* in mixed or unialgal cultures.

Sewage plant effluents, even with an optimum combination of unit treatment processes, normally contain fragments of the original waste components. With population densities increasing rapidly and with the subsequent re-use of water, a stream may in time contain rather high concentrations of nutrients which in turn will stimulate intense algal growths where environmental conditions permit.

It would seem logical that if algal blooming is a result of nutrient discharge, this phase of biological assimilation could possibly be added as a tertiary stage of sewage treatment and kept within the confines of the sewage treatment plant. The objective of such a process would be to absorb the soluble nutrients by means of an actively metabolizing algal culture and then to

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remove the algae completely from the effluent before releasing it to the receiving waters.

Whether this objective can ever be realized depends first on the accumulation of sufficient knowledge concerning the life processes of useful algal species, and secondly upon man's ability to provide the required environment necessary to assure the successful growth of such species under artificial conditions.

The use of algal cultures to enhance the removal of organic constituents from sewage has had wide application in the form of lagoons or sewage ponds. An excellent review of the present information available in this area is afforded by the recent article of Fitzgerald and Rohlich.[1]

Present studies at the University of Michigan have been in progress since the fall of 1956, and are directed specifically towards the controlled use of algae in the sewage treatment cycle in contrast to the use of sewage lagoons which must be classed as an uncontrolled use.

As a first step towards the attainment of the objective of algal assimilation, batch cultures of local river water mixed with activated sludge effluent were incubated at 20°C under a light intensity of 200 ft cd. Results from these studies indicated that *Scenedesmus*, *Ankistrodesmus* and *Chlorella* were the organisms most frequently produced. These algal species were isolated and used in subsequent experimental work. (Fig. 1.)

During the research programme, no attempt was made to maintain aseptic conditions since, in the event of the practical application of algal culture to sewage treatment, aseptic conditions would not be feasible. In the initial phases of the research a unialgal culture of *Chlorella* was the test organism; however, the culture tended to revert to a mixture of *Scenedesmus* and *Chlorella* with *Scenedesmus* predominating. This mixed culture has been under continuous test for a period of more than a year with little, if any, difficulty experienced in its maintenance.

The effects of light quality and intensity on algae have been widely considered by other investigators.[2–5] Utilizing available data, a light source was constructed which would provide a major portion of its relative energy spectrum in the 400–700 mμ range and with intensities varying from 100 to 2,000 ft cd.

Shading effects were held constant by construction of a culturing

device which poised the algal cell count at a definite level for a complete series of runs. This was accomplished by the use of light-sensitive cells placed on the back of the culture chamber (Fig. 2). Any change in transmittance of the culture due to growth actuated the photocells which, in turn, diluted the culture to the original concentration with fresh substrate. The volume of mixed liquor displaced was plotted automatically against time to provide a continuous visual curve showing the growth rate.

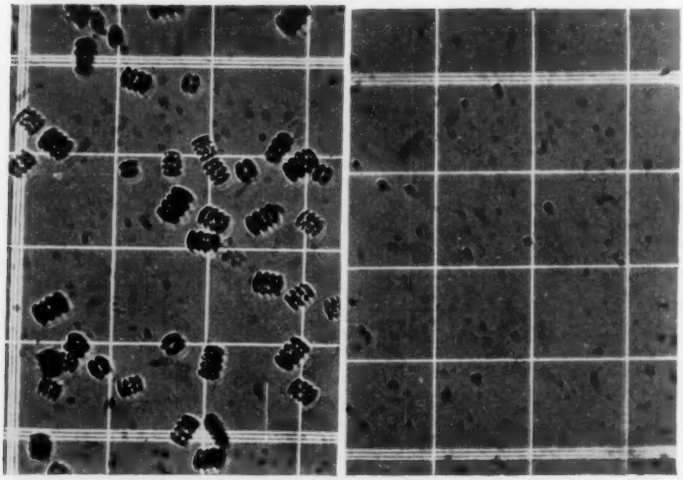

Fig. 1. (Left) *Scenedesmus* and (right) *Chlorella*; hemocytometer cell with markings at 50 μ intervals

The initial volume of the culture was 3·4 l., and the thickness 5 cm.

In order to avoid inconsistencies due to slight changes in turbidity of the nutrient solution or changes in the light intensity due to line voltage variations, the differential photocell circuit of Wood, employing a control chamber, was used.[6] The entire apparatus was enclosed in a room in which the temperature could be varied from 15 to 30°C $\pm$ 0·5°.

The source of nutrients was secondary effluent from an activated sludge plant. A sufficiently large batch was obtained each

time a run was contemplated, so that little change in the substrate occurred while data were being recorded.

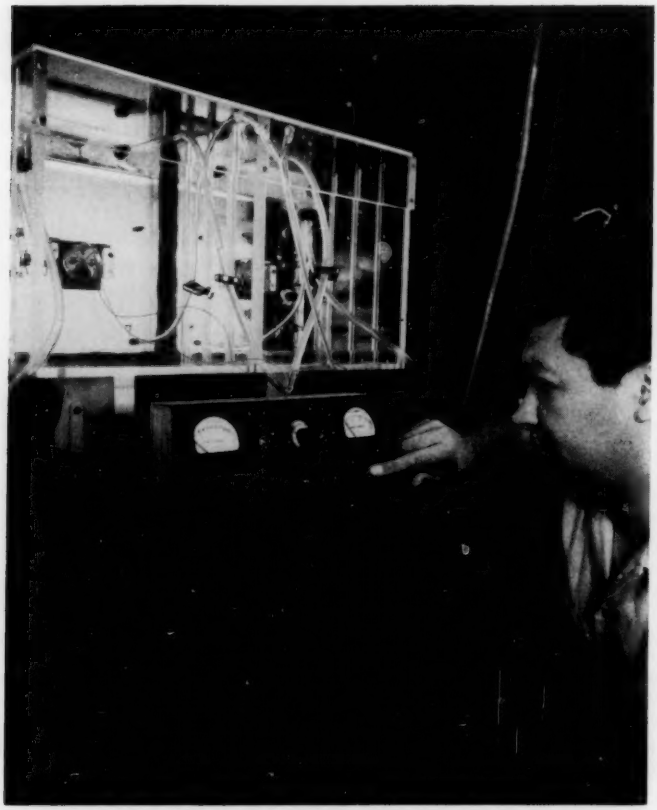

Fig. 2. Rear view of culture chamber showing photocells

As a supplement to the normal sewage nutrients, carbon dioxide was fed as a 5 per cent CO_2–95 per cent compressed air mixture. In most runs a rate of 1 to 5 ml per min proved entirely adequate for peak growth. Investigation to date has centred around two main objectives; first, the rate of growth of the test organisms

utilizing sewage nutrients, and, secondly, the rate of removal of such nutrients.

Rate of growth has been measured by algal count and net cell volume, packed cell volume, dry weight and density, by optical density, and simply by displaced volume of mixed liquor.

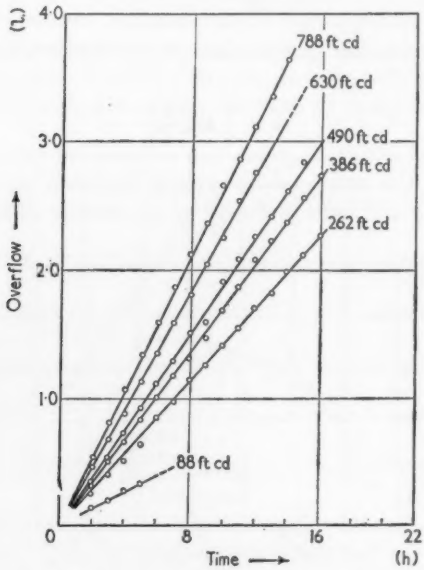

Fig. 3. Accumulated culture overflow as a function of time for various light intensities (Run Ia, 20°C)

Of the techniques enumerated, displaced volume seemed the most logical since it proved to be the simplest measurement to make and it was found that cell size, as well as cell density, apparently varied widely as environmental parameters were changed.

Fig. 3 shows the results of an early run with the equipment, and clearly illustrates the stability and sensitivity of the system as developed using the displaced volume as a measure of growth rate. This graph also demonstrates the suitability of secondary sewage effluent as a substrate.

With all the important variables kept constant and with the growth established at a steady rate, it becomes possible to express the growth rate k in terms of two parameters, namely, time and culture volume, through the following differential equation:

$$\frac{dV}{dt} = kV$$

From this expression integration yields the following solution for k:

$$k = \frac{1}{t} \log \frac{V_2}{V_1}$$

Fig. 4 shows the relationship existing between growth rate as expressed by k and light intensity at the surface of the culture.

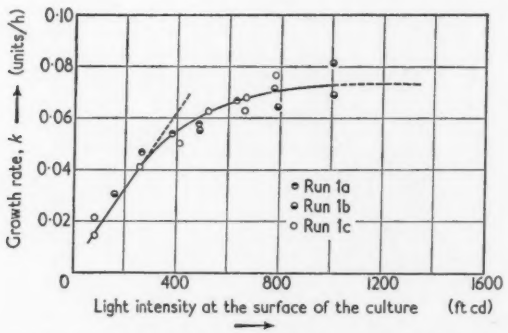

Fig. 4. Growth rate of *Chlorella* as a function of light intensity in secondary effluent (20°C)

Reference to Fig. 4 indicates that at low light intensities, with all other independent variables controlled, a direct proportionality between growth rate and light intensity is apparent. With a further increase in light intensity, the rate of change of growth rate is reduced and at high light intensities an adverse or toxic effect is produced. These results have been observed by other research workers studying the production of algae using synthetic media.

It is interesting to note that, in comparison to synthetic media,

secondary sewage effluent appears to be a very acceptable substrate. This fact is indicated by a comparison of growth rate curves. Both shape of curve and peak rate of growth appear to be virtually the same when either medium is used.

Temperature effects are shown in Fig. 5 for the range between 20° and 30°C. Little, if any, effect on growth rate is apparent at low light intensities. At moderate to high light intensities, however, the light saturation intensity level (that intensity level at which the maximum rate of growth is attained) increases considerably with each five degree increase in temperature. There

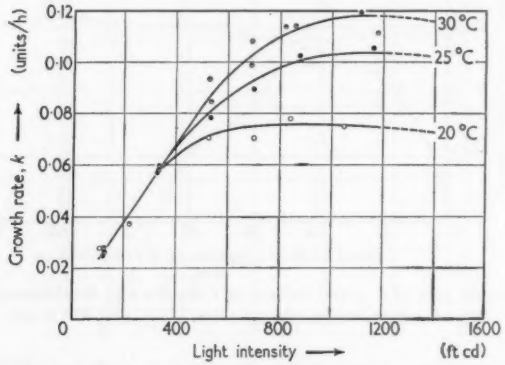

Fig. 5. Growth rate of a mixture of *Chlorella* and *Scenedesmus* as a function of light intensity at various temperatures

is evidently an intimate relationship between temperature and light intensity. This function is being given more adequate study.

Cell count or concentration of organisms is an important factor in the metabolism of algae. Fig. 6 indicates the change in growth rate experienced as the culture transmittance decreases. Above the level of 60 per cent transmittance the growth rate appears to be relatively independent of the number of organisms. Whereas, below this point growth rate is seriously impaired by an increase in cell concentration.

Such a drop in growth rate may be due to any one or a combination of several factors. It is possible that the cells are

experiencing periods of reduced light exposure, or that inhibitive competition exists, or that toxic products are being formed. Further study of varying depths of culture at varying nutrient levels is necessary to define this relationship more clearly.

The carbon content of secondary sewage is also quite a serious problem in relation to cell concentration. *Scenedesmus* readily utilizes the bicarbonate form of alkalinity and thus tends to increase the pH of the culture. Good growth was observed at all

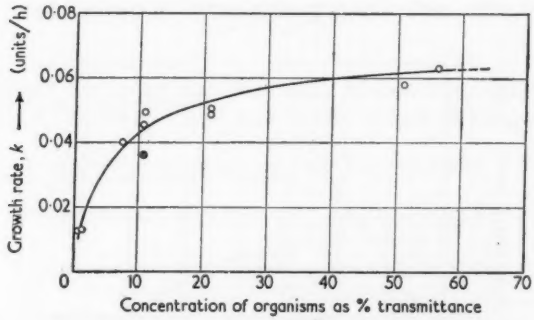

Fig. 6. Growth rate of a mixed culture of *Chlorella* and *Scenedesmus* as a function of concentration of organisms (20°C and 670 ft cd)

pH levels from 6·0 to 9·5 with the best growth at pH 8·3. The secondary sewage used as substrate had a total alkalinity averaging 120·0 ppm as $CaCO_3$ (Table I) and an ammonia nitrogen

Table I. Composition of activated sludge effluents used in these experiments

	Alk	NH_3	NO_2	NO_3	T.I.N.	PO_4
Series I	204·6	23·3	0·47	0·18	23·98	8·7
Series III	181·0	20·5	1·20	1·58	23·27	9·0
Series IV	120·0	15·96	1·27	7·80	24·99	20·0

content of 16 ppm as N. This type of substrate was capable of supporting 1,000 *Scenedesmus* cells per mm^3. Beyond this level carbon became limiting and an additional amount was supplied

in the form of carbon dioxide. Fig. 7 shows a typical run at 1,500 *Scenedesmus* cells per mm³. The slope of the curve indicates the rate of growth which is steady at point A. When the CO_2 source is shut off, the culture gradually adapts itself to the bicarbonate as a carbon source (point C), and subsequently depletes this reserve before growth ceases at about pH 10·0 (point E).

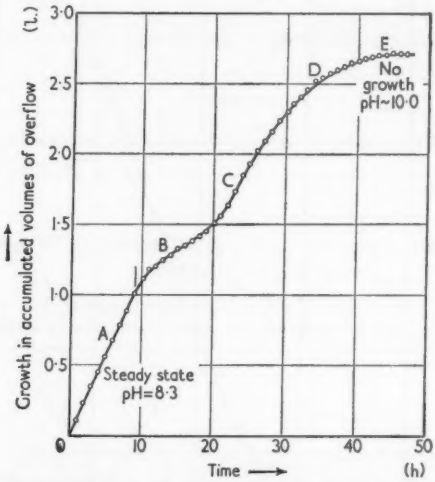

Fig. 7. Changes in the growth of a culture of *Scenedesmus* when available carbon is less than required (20°C, 660 ft cd and 1,500 org./mm³)

Fig. 8 shows the growth pattern of a culture containing 12,000 *Scenedesmus* cells per mm³. This culture was aerated with 100 per cent carbon dioxide until growth ceased at pH 5·5. When the carbon dioxide feed was stopped the culture gradually resumed growth, utilized all the free and half-bound carbon, and finally the growth again ceased at a pH of 11·0. The lack of a lag phase in this case between the metabolism of free carbon dioxide and the bicarbonate may be attributed to the extreme competition in this rather dense culture.

Concentration of organisms also seems to play an important part in the efficiency of both production of cells and nutrient removal. Figs. 9 and 10 illustrate this point. Both the weight

and volume of algae produced are a maximum at one level of energy absorption. The removal of inorganic nitrogen from the medium is also optimum at or near this same level. Since absorption of the incident light is a function of the concentration

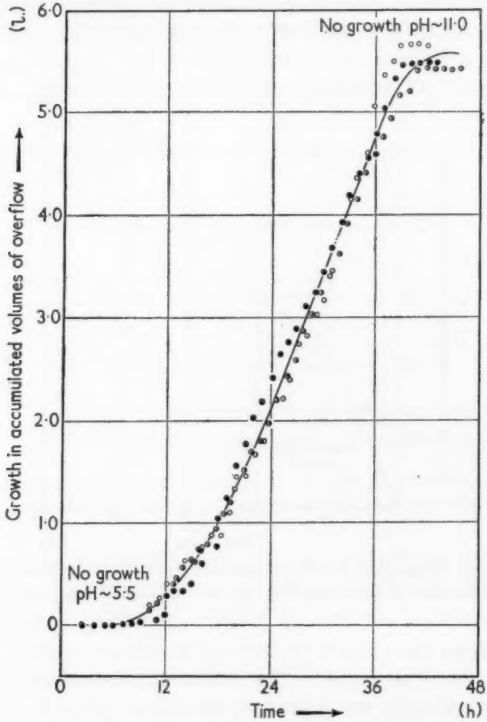

Fig. 8. Changes in the growth of a culture of *Scenedesmus* with depleting carbon available (25°C, 1,050 ft cd and 12,000 org./mm³)

of the organisms, it may be concluded that there is one optimum level of cell concentration, other factors being constant, which will produce the most effective nutrient removal.

With the equipment and culture being used, the best removal came at 9,000,000 cells per ml. This organism concentration corresponded to an optical density of 90 per cent. Reference to

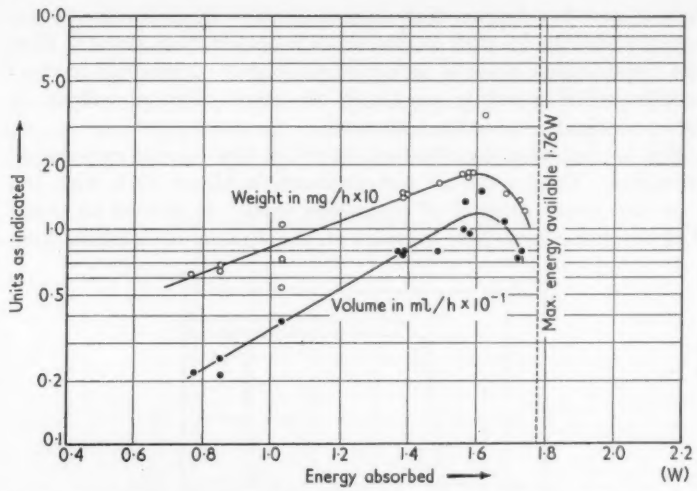

Fig. 9. Weight and volume of algae produced in a mixed culture of *Chlorella* and *Scenedesmus* as a function of the energy absorbed (20°C, 670 ft cd)

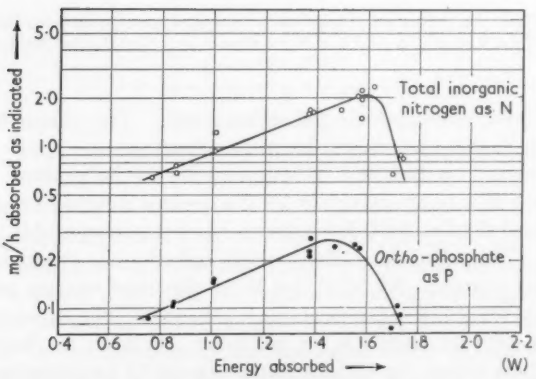

Fig. 10. Inorganic nitrogen and *ortho*-phosphate removed in mg/h by a mixed culture of *Chlorella* and *Scenedesmus* as a function of the energy absorbed (20°C, 670 ft cd)

Figs. 5 and 6 indicates that such a level of light transmittance would probably be well below the light saturation point. This fact implies that there is an optimum degree of shading or competition which actually results in the most effective culture as far as nutrient removal is concerned.

Fig. 11 indicates the effect of detention time on the removal of nutrients. Peak removal was attained in about 25 h with the type and concentration of organisms used. It should be noted that detention time here is based on the volume of overflow from

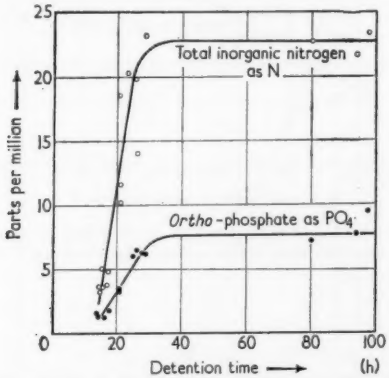

Fig. 11. Parts per million of nitrogen and phosphorus removed from the effluent by a mixed culture of *Chlorella* and *Scenedesmus* as a function of detention time (20°C, 670 ft cd)

a well-mixed, continuously fed culture cell. The effects of short circuiting in this case would be pronounced.

With regard to the effect of temperature on the process, Fig. 12 shows that at a temperature of 25°C a greater weight of organisms is produced than at 20°C for cultures having a comparable number of cells. This effect is made possible only by the expenditure of additional energy. At 20°C, 1·4 W of absorbed energy produced 15 mg dry weight of algae per hour while, at 25°, the same expenditure of energy produced 9 mg per hour. Within the limitations of the data taken up to this time the 20°C temperature level appears to be the most effective for nutrient removal.

The maximum dry weight production of organisms at 25°C

was about 30 mg/h. It is interesting to compare the maximum yield of cell material in these experiments with the values obtained by Ludwig *et al.*[7] This author, under the same light and temperature conditions, cultured *Euglena gracilis* using settled raw sewage and supplemental carbon dioxide as a substrate. The reported yield was 0·26 gm/l. day which compares within 4 per

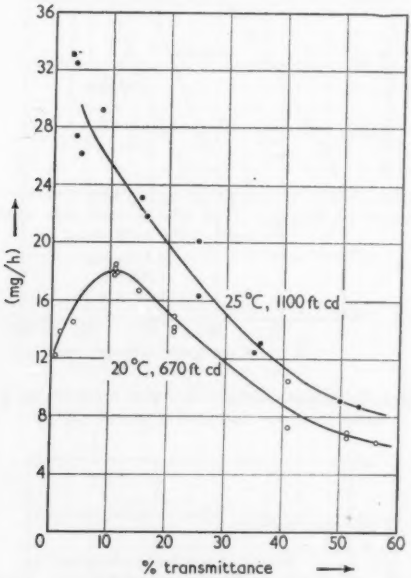

Fig. 12. Weight of organisms produced by a mixed culture of *Chlorella* and *Scenedesmus* as a function of transmittance

cent the yield reported here in secondary sewage. The implications of this fact would be that there are growth stimulating ingredients which may pass through a secondary treatment plant and which, to a large extent, negate the added degree of treatment as far as the production of certain algal forms in the receiving water is concerned. This assumes that a plentiful supply of carbon is readily available.

Fig. 13 shows the percentage of each form of inorganic nitrogen

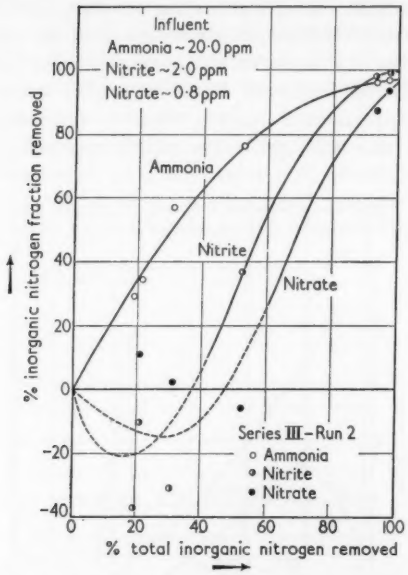

Fig. 13. Percentage inorganic nitrogen fraction removed as a function of the total inorganic nitrogen removed (20°C)

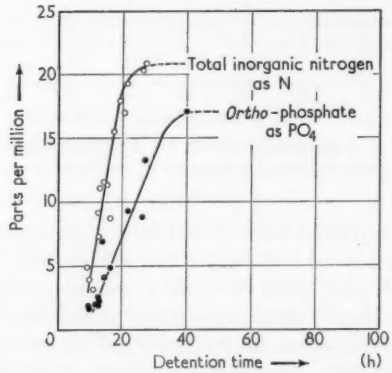

Fig. 14. Inorganic nitrogen and *ortho*-phosphate removed from the effluent by a mixed culture of *Chlorella* and *Scenedesmus* as a function of detention time (25°C, 1,050 ft cd)

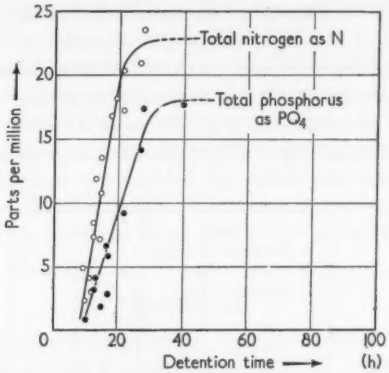

Fig. 15. Total nitrogen and phosphorus removed from the effluent by a mixed culture of *Chlorella* and *Scenedesmus* as a function of detention time (25°C, 1,050 ft cd)

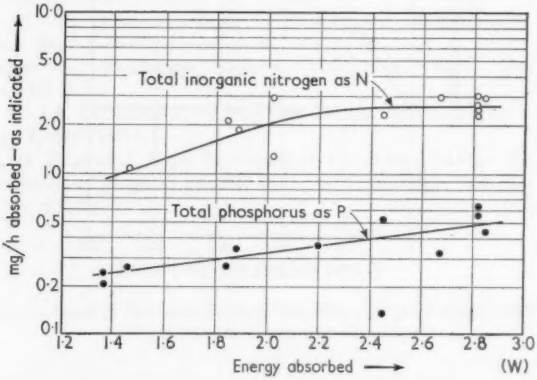

Fig. 16. Inorganic nitrogen and *ortho*-phosphate removed by a mixed culture of *Chlorella* and *Scenedesmus* as a function of energy absorbed (25°C, 1,050 ft cd)

removed. These data indicate that the ammonia nitrogen is the first form of nitrogen attacked. Figs. 14 and 15, when contrasted, indicate that the organic nitrogen fraction is probably attacked after the inorganic nitrogen and at the same rate. Fig. 11 indicates that the same rate of removal (in ppm) prevails

at 20°C as at 25°C (Fig. 14). It should be noted that this is not true in the case of the removal of the phosphorus compounds.

Comparison of Figs. 10 and 16 would indicate that the efficiency of removal of inorganic nitrogen is somewhat higher at the lower temperature. Reference to Fig. 17, in contrast to Fig. 13, shows that the ammonia nitrogen is just about completely removed from

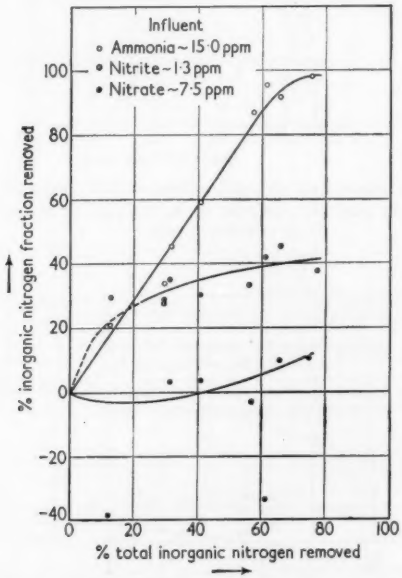

Fig. 17. Percentage inorganic nitrogen fraction removed as function of the total inorganic nitrogen removed (25°C)

the substrate. However, nitrites and nitrates are more slowly metabolized and, especially at the 25°C temperature level, are absorbed very ineffectively.

In contrast to nitrogen, both inorganic and organic phosphorus appear to be absorbed at the same time. In addition, phosphorus is removed more effectively at 25°C than at 20°C. While phosphorus was never completely absorbed from the substrate, its removal continued long after the absorption of nitrogen had

ceased. This fact would indicate storage of this element within the cell.

The observations reported on above indicate that good efficiency of removal of ammonia nitrogen can be expected within relatively short detention times. This compound appears to be found more frequently in high quantities in modern secondary sewage treatment effluents than is anticipated by many operators who fail to even test for it. Tertiary algal treatment is a possible technique for removal of this nutrient from the waste before discharge.

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A Conductance Bridge Method for the Amperometric Estimation of Low Oxygen Tension

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Summary. Oxygen tension in aqueous media of the order of 10^{-6} to 10^{-9} molar is measured amperometrically by means of a stationary inert metal electrode. The electrolytic cell is connected into a high resistance bridge circuit, which is energized from a low resistance d.c. source of voltage adjusted to give a potential difference across the cell corresponding to a position on the diffusion current plateau of the first oxygen wave. This cell potential is monitored by means of a vacuum tube millivoltmeter and maintained constant. A vacuum tube millivoltmeter measures the bridge output. The bridge is initially balanced with the medium deoxygenated; subsequent introduction of low concentrations of oxygen dissolved in the medium unbalance the bridge. Either the resulting bridge output voltage, as read on the millivoltmeter, or the resistance change in the bridge arm opposite the cell, is related to the oxygen tension by means of a previous calibration. Both quantities are directly proportional to oxygen concentration, at least over limited ranges of such concentration changes. The method is also applicable to the estimation of other substances which can be discharged under amperometric conditions at a stationary electrode.

The requirement to measure accurately very low concentrations of oxygen dissolved in an aqueous medium arises frequently in biological studies. Amperometry with a rotating gold or platinum electrode has been applied to the measurement of oxygen in stirred microbial culture.[1] The rotating electrode is useful in the range above 10^{-6} M, when the pH of the culture is not less than about 5; however, optimal growth often requires that the pH be less than 5; also oxygen requirements of cultures may be satisfied by concentrations below 10^{-6} M. Polythene

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membranes allow free diffusion of oxygen but not of hydrogen ion; hence, by covering the electrode with polythene, oxygen may be measured in cultures at very low pH.

Polarographic methods, using a gold electrode as the cathode[2] become increasingly inaccurate and unreliable as oxygen tension is reduced below about 10^{-6} M, since then the diffusion current due to the reversible discharge of oxygen tends to become comparable with the residual current. Increase in the electrode area does not improve the method, since the ratio between the residual and the diffusion current remains unaltered.

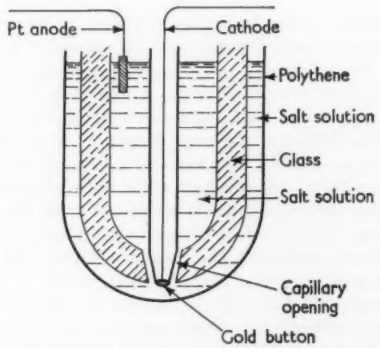

Fig. 1. Gold electrode for oxygen determinations (after Charlton, reference 3)

A gold electrode covered with a semipermeable polythene membrane[3] was found to operate well at higher values of oxygen concentration and attempts were therefore made to devise means to allow the use of this electrode at oxygen concentrations below 10^{-6} M. A diagram of the type of electrode used[3] is shown in Fig. 1.

While it is possible to compensate the residual current by the introduction of an opposing current of appropriate magnitude, such a method would involve the measurement of a small difference between the values of two large currents, and thus would require a compensating current source of very high stability indeed. It thus appeared to be preferable to measure the conductance of the electrode system rather than the current passing.

Using a dropping mercury electrode, Ilkovič and Semerano[4] have employed a bridge circuit to balance out the residual current in conventional d.c. polarography, allowing the cell potential to find its own level. In the present method, the potential difference across the electrodes is maintained constant throughout at a value corresponding to the diffusion current region, which was located from the results of an initial measurement, as proposed by Petering and Daniels, and by Baumberger.[5]

Applying the conventional d.c. polarographic technique to the electrode system yielded the current–voltage curve shown in Fig. 2. This refers to 300 ml of deoxygenated distilled water,

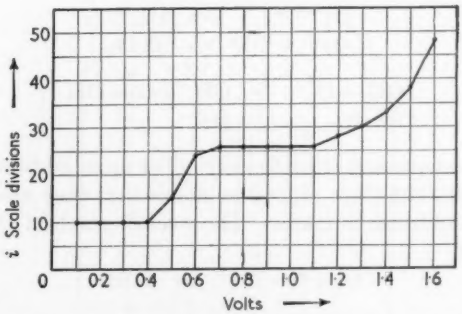

Fig. 2. D.C. polarogram of 95% oxygenated water, obtained with the electrode shown in Fig. 1

(prepared by thorough flushing with nitrogen), to which were added 40 ml of 95 per cent oxygenated water, yielding an oxygen concentration of $1{\cdot}26 \times 10^{-4}$ M. The reference electrode was platinum foil in contact with a saturated solution of potassium chloride. The active electrode consisted of a gold button of 20 mm^2 area; the entire electrode assembly is shown in Fig. 1.

It is seen from Fig. 2 that the current, once having reached the diffusion current plateau, remains substantially constant and is little affected by the exact value of potential, within the region of 0·7 to 1·1 V. By selecting a value of potential near the middle portion of the diffusion current plateau, none of the distortions of the polarographic curve noticed by Longmuir[6] were encountered.

The polarographic discharge of oxygen at a dropping mercury

electrode was studied by Kolthoff and Miller,[7] who ascribed the first oxygen wave to the reaction

$$O_2 + 2e + 2H^+ \rightarrow H_2O_2 \qquad (1)$$

and by Hacobian,[8] who postulated a one-electron process involving either the oxygen molecule or the oxygen atom.

However, the electrochemical conditions at a gold electrode differ from those obtaining at a dropping mercury electrode, and Kolthoff and Jordan[9] proposed the following electrode reactions for the discharge of oxygen at a gold electrode:

$$\left.\begin{array}{ll} O_2 + e & \rightarrow O_2^- \\ O_2^- + e & \rightarrow O_2^{--} \\ O_2^{--} + 2H^+ & \rightarrow H_2O_2 \end{array}\right\} \qquad (2)$$

$$\left.\begin{array}{ll} H_2O_2 + O_2^- & \rightarrow OH^- + O_2 + OH \\ OH + e & \rightarrow OH^- \end{array}\right\} \qquad (3)$$

The steps in equation (3) definitely occur upon addition of hydrogen peroxide, but there is good evidence that they also take place if no such addition is made. The overall reaction thus appears to be:

$$O_2 + 4e + 2H^+ \rightarrow 2OH^- \qquad (4)$$

The half-step potential for the first oxygen wave at a platinum electrode, is given in the literature[1] as $-0{\cdot}680$ V against the standard hydrogen electrode; under our experimental conditions it is seen that the half-step potential is located at $-0{\cdot}525$ V. The potential appears to be shifted to less negative values due to the lower oxygen over-voltage of oxygen on gold. The second oxygen step merges with the discharge of hydrogen.

The electrode system was connected into the bridge circuit shown in Fig. 3. The bridge supply voltage was varied by means of the 100-Ω potentiometer (P) until the vacuum tube voltmeter, connected across the cell, indicated a potential difference corresponding to the diffusion current plateau as located in the initial experiment; a value of 0·95 V was found to be satisfactory. The solution in the cell was deoxygenated distilled water. The bridge was then balanced by varying the value of one of the ratio arm resistances, D, until stable and accurate null balance was obtained.

The residual conductivity of the electrode system was thus balanced out. On the introduction of small measured quantities of oxygen into the solution, the system behaves like a conventional amperometric circuit, giving rise to a diffusion current which is proportional to the oxygen concentration. The flow of this diffusion current, however, means that the cell conductivity has slightly increased, unbalancing the bridge. If the oxygen concentration is small, the resulting unbalance current may be

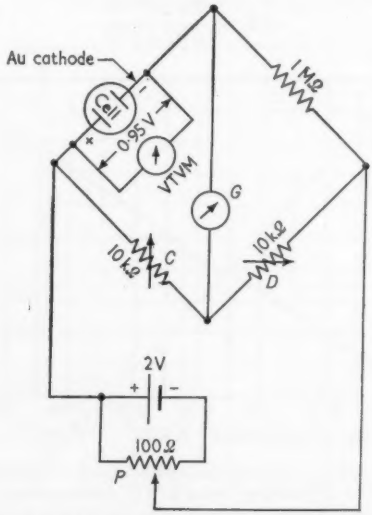

Fig. 3. Conductance bridge circuit for amperometric oxygen determinations

read directly from the millivoltmeter G without rebalancing the bridge. If, however, the range of oxygen concentration is large, then it is preferable to rebalance the bridge, say by varying the setting of the ratio arm D, until a balance has been re-established for a given value of oxygen concentration. Departures from this new fiduciary value are then read from the millivoltmeter G. Alternatively, its sensitivity may be reduced to bring the reading onto the scale. It is thus recommended to use the circuit as direct reading only over an approximately twenty-fold range of oxygen concentrations, changing the range by rebalancing the

bridge. Over a twenty-fold range, a nearly linear relationship between the unbalance current, as indicated by the millivoltmeter, and the oxygen concentration in the solution, is obtained, as seen from Fig. 4.

This output voltage ΔE of the unbalanced bridge shown in Fig. 4, assuming the departure from balance, i.e. the change in the oxygen concentration from that at which the bridge was initially balanced, is given by

$$\Delta E = \frac{E_0 \Delta X D^2}{S(C+D)^2} \tag{5}$$

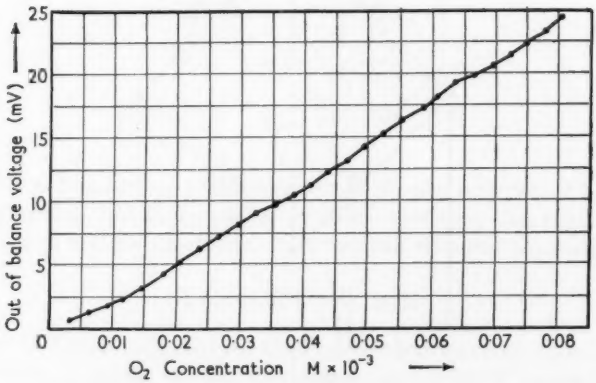

Fig. 4. Calibration curve of the unbalanced bridge shown in Fig. 3. The bridge is initially balanced at a fiduciary value of O_2 concentration. The calibration curve shown indicates the output voltage of the unbalanced bridge as a function of subsequent changes in O_2 concentration

where E_0 is the bridge supply voltage, 0·963 V in the present case; ΔX the change in the equivalent electrode resistance due to the electrochemical process; and S the value of the standard resistance in the bridge, i.e. 1 MΩ in the present case. C and D are the ratio arms, as shown in Fig. 3. The input resistance of the vacuum tube millivoltmeter must not be appreciably less than the bridge resistance. Alternatively, the bridge output current may be measured by means of a mirror galvanometer.

Greater accuracy and an extended range of linearity to oxygen concentration are attainable if the bridge is rebalanced after each

change in oxygen concentration. Since a conductance change in one arm of a bridge is compensated by a resistance change in the opposite arm, the value of the resistance D required to balance the bridge will be linearly related to the change of the cell conductivity, and thus to the diffusion current and to the oxygen concentration. This is illustrated in Fig. 5, which shows a plot of the value of the resistance D *versus* oxygen concentration from 3×10^{-6} to $108{\cdot}8 \times 10^{-6}$ M. The behaviour of the system as depicted in Figs. 1 and 3 is summarized in Table I.

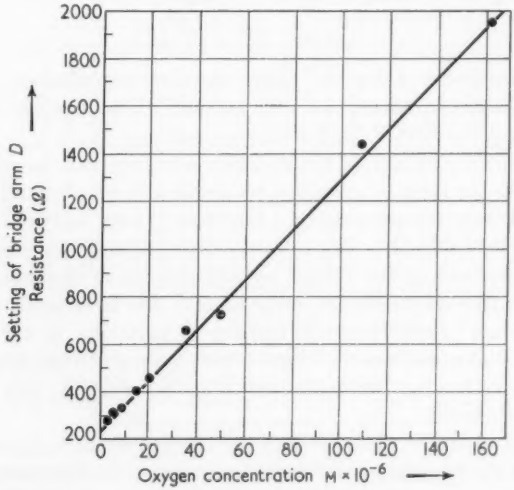

Fig. 5. Calibration curve of the balanced conductance bridge. Balance is restored for each value of O_2 concentration by varying the bridge arm 'D' in Fig. 3. The calibration curve now relates the value of the resistance 'D' to O_2 concentration

Once a calibration of the device has been obtained from a series of measurements on solutions of known oxygen concentrations, the oxygen concentration in a medium may be accurately and continuously measured. The lower limit of sensitivity of the method to the presence of dissolved oxygen in an aqueous medium was found to be about $4{\cdot}5 \times 10^{-9}$ M, using a Philips type M 6010 electronic d.c. millivoltmeter, having a sensitivity of 0·01 mV per division and an input resistance of 0·67 MΩ.

Table I. Parameters of the method

Cathode area = 0·2 cm²
Residual current = $1{\cdot}3 \times 10^{-8}$ A or $6{\cdot}8 \times 10^{-8}$ A/cm²
Residual cell resistance = 73·5 MΩ
Detector sensitivity = 0·01 mV per scale division, input resistance 0·67 MΩ
Minimum detectable oxygen concentration = $4{\cdot}5 \times 10^{-9}$ M
Voltage sensitivity of bridge to oxygen = 0·85 mV/10^{-6} M oxygen

A change in oxygen concentration of the medium of 3×10^{-6} M causes the following changes:

Change in cell resistance	3 MΩ
Change in the setting of arm D	26 Ω
Voltage output of bridge	2·5 mV

The electrode assembly used in our experiments initially required approximately 45 min to come into equilibrium after connecting the 0·95 V bridge supply voltage.

The reproducibility of the resistance change due to addition of 1 ml of 95 per cent oxygenated water to 350 ml of an oxygen-free standard sucrose–ammonium-phosphate yeast nutrient medium is shown in Table II. Ten minutes were required for stabilization before rebalancing the bridge by adjustment of the resistance D. The variation of the initial value of D is due to adjustment of the ground joint of the electrode leading to variation in the distance between the membrane and gold button in different tests. It is important that no further adjustment be made to the electrode after calibration.

A large change in the value of the bridge resistances does require a change in the setting of P in order to maintain the voltage across the cell at its required value; however, for small variations in D, as required for balancing out the residual conductivity of the

Table II. Reproducibility of the method

Procedure:—Values of the resistance D in Fig. 3 required for balance

350 ml bacterial culture medium, oxygen-free	370	320	252	270	271	270
1 ml of 95% oxygenated water added	380	328	268	278	279	278
Test number	1	2	3	4	5	6

system, no variation in the cell potential was observed so that the setting of P need not be varied.

The applicability of the method is clearly not confined to the measurement of oxygen concentrations, but may be applied to the amperometric estimation of any other substance, which may be discharged under the conditions described. This is illustrated in Fig. 6 which shows a plot of unbalance voltage *versus* concentration of Tl^+ in TlCl, the potential across the cell being maintained at 1·05 V, corresponding to the diffusion current plateau located in a preceding experiment.

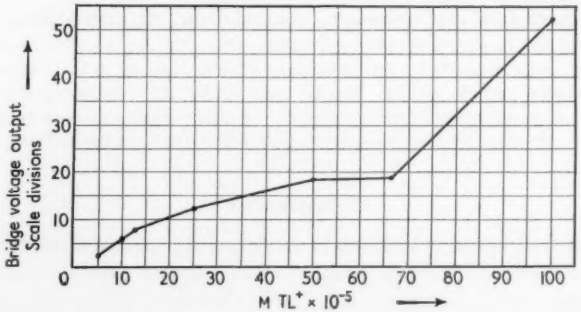

Fig. 6. Use of the conductance bridge of Fig. 3 for amperometric estimations of substances other than O_2. This calibration curve relates the output voltage of the unbalanced bridge to the concentration of Tl^+, and is analogous to the O_2 calibration curve shown in Fig. 4

The method, being continuously direct reading, should lend itself readily to continuous control and automatic recording. However, attention should then be given to the amount of oxygen consumed by the method itself: $1{\cdot}2 \times 10^{-14}$ moles of oxygen are reduced per second under the experimental conditions described.

A method of continuous oxygen measurement, similar to that described, is at present in operation in our laboratory to control dissolved oxygen in continuous flow microbial cultures.

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Polarographic Measurement of Oxygen Supply and Demand during Aerobic Propagation of a Bacterial Culture*

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Summary. The sampling method of polarographic analysis for dissolved oxygen in culture medium is unaffected by the presence of viable micro-organisms. This method allows measurement of peak oxygen demand, critical oxygen concentration, and oxygen supply during actual mass propagation of an aerobic culture. Fermentation operating conditions may be adjusted to supply the oxygen required by the aerobic culture.

Introduction

The efficient mass propagation of aerobic cultures demands an adequate oxygen supply; the rate of supply of oxygen must balance the demand rate of the culture. Aerobic micro-organisms respire by using only dissolved oxygen[1, 2] and oxygen is rather insoluble in liquid media (0·21 millimoles of oxygen per litre of water at 37°C, atmospheric pressure). The rate of supply of oxygen from the sparged air to the culture is limited by the low solubility of oxygen and need only be that required to maintain the minimum level of dissolved oxygen demanded by the culture.[1–3] This minimum level is termed the critical oxygen concentration and never approaches the saturation solubility of oxygen. The critical concentrations for aerobes vary in the range of 0·003 to 0·050 millimoles of oxygen per litre, depending on the culture, medium and environmental conditions during propagation.

The general picture of oxygen demand and supply in aerobic cultures is described by Finn[1] and Steel[2] and the basic rate equations described there are followed in this paper.

* Presented at the Fermentation Subdivision Meeting of the American Chemical Society, Atlantic City, September 1959.

The sampling method of polarographic analysis, in which a continuous sample of culture is transferred through the polarographic cell, has a number of advantages over the 'gassing out' method.[4] In addition to measuring the rate of supply of oxygen to the culture, the air supply to the culture trapped within the polarographic cell may be cut off and the rate of oxygen demand by the culture may be measured from the time-rate of decrease in dissolved oxygen. Oxygen uptake by a culture has been shown to be independent of dissolved oxygen concentration above a certain critical oxygen concentration and this critical concentration of dissolved oxygen may also be measured.[1-3] Since the supply and demand of oxygen to an aerobic culture can be measured, the oxygen absorption coefficient may be calculated and the vessel operating variables may be adjusted so that the supply of oxygen exceeds the maximum culture demand. Since the rate of supply of oxygen, in a steady state, must equal the rate of demand by the respiring culture:[1, 5]

$$C_c Q_{O_2} = k_L A (C_{Sat} - C_L) \tag{1}$$

$$k_L A = \frac{C_c Q_{O_2}}{(C_{Sat} - C_L)} \tag{2}$$

where C_c denotes concentration of viable cells (viable cell count per litre of medium), and Q_{O_2} is the specific oxygen demand rate (millimoles of oxygen per cell per minute).

The work reported here was undertaken as a follow-up to laboratory studies by Warshowsky and Schantz[6] of the measurement of dissolved oxygen during propagation of *Serratia marcescens* cultures. The continuous sampling method of polarographic analysis was used in our laboratory to measure respiration rates and the critical dissolved oxygen concentration for *S. marcescens* cultures during propagation in an agitated, sparged fermentor vessel. The oxygen absorption coefficient has been determined for the fermentation system.

Materials and Methods

The components of the polarograph system include: a small, non-polarized, rotating platinum electrode; a large, polarized

silver–silver chloride reference electrode; and an amplifying, recording microammeter (see Fig. 1).

The rotating platinum electrode unit consists of a platinum electrode driven through a belt and pulley system by a 1/20 h.p., totally enclosed, induction motor at 600 rev/min (see Figs. 2 and 3). The electrode was constructed by using a platinum wire of approximately 0·5 mm diameter inserted into a glass tube with an i.d.

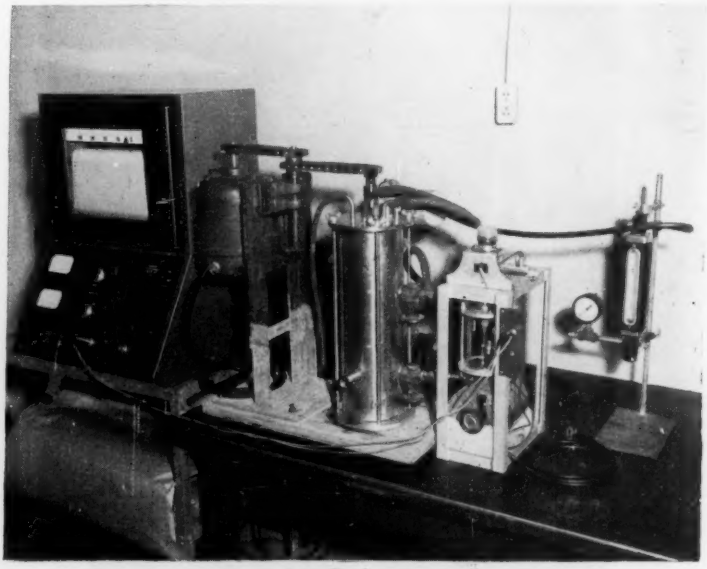

Fig. 1. Polarograph system

of approximately 2 mm. The wire is held in position by sealing wax. The tip of the wire protrudes from the glass tube a distance of 3 mm. The tip is bent at a 90° angle with respect to the axis of the tube. At the other end of the tube, the wire protrudes a distance of approximately 1·5 in. The electrode is inserted into a chuck and connected by a hollow shaft to a pulley. The tip of the glass tube is mounted flush with the upper end of the hollow tube which extends into a mercury pool chamber. The 1·5 in. extension at the end of the tube is bent over and is immersed in

the pool of mercury. The pool for the mercury is made of Teflon with an aluminium housing which is electrically insulated from the frame of the electrode unit. A hole was drilled and tapped through the aluminium housing and into the mercury pool. A screw was inserted to provide electrical contact between the mercury pool

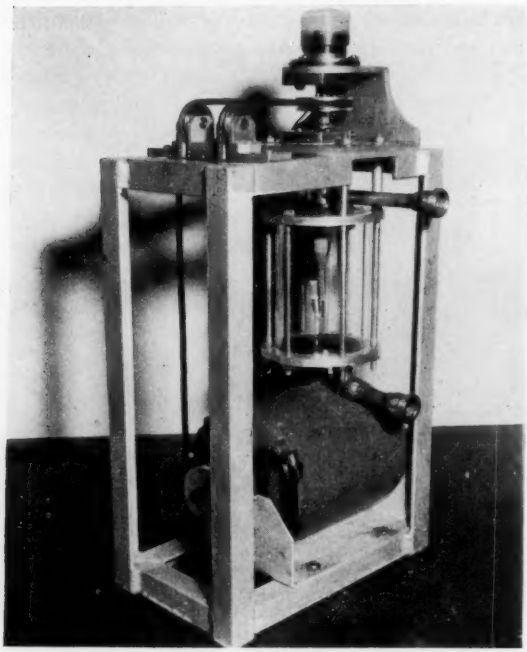

Fig. 2. Polarographic sampling cell

and the metal housing. A Lucite cap was provided for the mercury pool to prevent the mercury from spilling.

The reference electrode is a silver billet style metallic electrode. The electrode was freshly coated with a silver chloride film before each laboratory fermentation. The reference electrode is mounted approximately an inch from the platinum electrode. A glass-walled, pressure-tight cell encloses both electrodes. The reference electrode is sealed to the upper support plate of the cell.

There is a jack mounted near the cell which connects the electrodes through a plug and cable to the amplifier.

A Sargent Model XXI Recording Polarograph was used for the amperometric measurement of the oxygen diffusion current. The operating procedures for the polarograph are described in the Sargent *Manual*.[7] The current sensitivity for these experiments

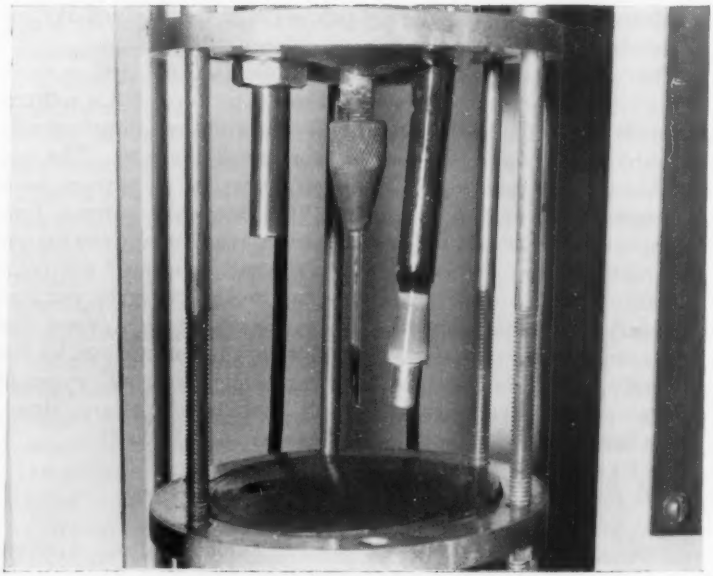

Fig. 3. Close-up of platinum and reference electrodes

was 0·1 microamperes per millimetre on the current axis of the chart. The chart speed was 8·0 seconds per unit on the time axis.

The fermentor was a 7·5 litre, agitated-air sparged vessel of conventional design. The glass tank was replaced with a 6-in. diameter stainless-steel tank. The tank was fitted with a gum rubber diaphragm and sampling adaptor[8] and was fitted with two Saunders-type diaphragm valve connections. The four-bladed, turbine impeller was located one impeller diameter above the tank bottom. The operating liquid volume was four litres. The

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fermentor temperature was controlled at 31 °C by an internal cooling coil. The agitation speed was calibrated with a stroboscope flash unit for various pulley combinations. Air flow was measured with a calibrated rotameter.

When a constant potential of −0·9 V (the potential of the diffusion current) is applied to the electrodes, the dissolved oxygen in the medium is reduced at the platinum electrode and the resulting current flow is directly proportional to dissolved oxygen concentration.[9]

The polarographic equipment was standardized before each series of observations. A standard salt solution of 0·5 M sodium chloride was added to the fermentor vessel and the equipment was operated as it would have been for a laboratory culture. The salt solution was saturated with oxygen from the air stream at a controlled temperature of 25 °C. The corrected current flow (sample reading minus the residual current reading) and the known saturated oxygen concentration for the salt solution[10] are combined to give a conversion factor of millimoles of oxygen per litre of solution per microampere. The polarographic equipment was also standardized periodically with sterile culture medium by the ascorbic acid oxidase method for determining dissolved oxygen in organic media. This method is fully described by Sharp, Hand, and Guthrie,[11] and is modified by Skerman and Millis.[12]

Experimental Procedure

The sampling method of polarographic analysis involves measurements of oxygen supply and demand during cultivation of *S. marcescens* 8UK in tryptose broth medium. All necessary precautions were taken to sterilize the equipment, prevent contamination of the culture and keep the medium aseptic prior to inoculation.

The tryptose broth medium (2% tryptose, 1% glucose, 0·5% sodium chloride) was prepared and sterilized within the fermentor vessel by autoclaving at 15 lb/in^2 gauge steam pressure for 30 min. The inlet and outlet sample valves to the fermentor were closed and the half-union connections to the polarographic cell were plugged with cotton and gauze. A small canister air filter, which was packed with glass wool, was sterilized with the fer-

mentor. When the fermentor unit was cool, the vessel was bolted to the supporting frame and the V-belt was attached between the motor drive and the agitator pulley. The agitator was started and the cooling water was admitted into the internal coil to hold the medium at 31°C.

The reference electrode was freshly coated with a silver chloride film and the platinum electrode was cleaned and polished. The glass polarograph cell was assembled and the inlet and outlet connections of the cell were plugged with cotton and gauze. The cell unit was sterilized by exposure to Carboxide gas at 10 lb/in^2 gauge pressure for 18 h.

The cell unit and fermentor unit were assembled by removing the cotton plugs on the connecting, stainless-steel half-unions; flaming and tightening the unions. The valve was opened below the medium level within the fermentor. As the medium flowed by gravity into the polarograph cell, a rubber hose loop in the medium return line was placed in a peristaltic pump. The pump provides an adequate circulation of medium within the cell and maintains the liquid level at 3 in. above the tips of the electrodes.

The 4 l. of tryptose medium in the fermentor were aerated with compressed air which entered the vessel through a single-orifice sparger after passing through a pressure regulating valve and rotameter at 4 l./min. The fermentor impeller was operated at 778 rev/min. The polarograph was started at an applied potential of $-0{\cdot}9$ V, 0·1 microampere per millimetre sensitivity and at maximum damping. At the saturation concentration for dissolved oxygen, the polarograph records a constant current flow. Cylinder nitrogen gas may then be substituted for air flow until the residual current level is reached. The polarograph will again record a straight line at a constant current flow.

If air at a measured flow rate is reintroduced into the fermentor (agitator speed constant), the time rate of increase in dissolved oxygen concentration from residual current to saturated current is recorded. The oxygen absorption coefficient and the oxygen supply rate for the fermentor may be calculated.

The fermentor medium was inoculated with 200 ml of a suspension of *S. marcescens* which contained 10^9 viable cells per millilitre. The aeration was maintained at 4 l./min, agitation at 778 rev/min, and temperature at 31°C for all laboratory cultures.

An actual polarograph curve, shown in Fig. 4, is typical of the oxygen demand curves observed for *S. marcescens* cultures.

Immediately after inoculation, the inlet and outlet valves to the polarograph cell were closed and a straight-line, time-rate decrease in dissolved oxygen was recorded. The slope of the straight-line, time-rate decrease in dissolved oxygen, in appropriate units, is the culture oxygen demand rate. The straight-line curve broke sharply at the critical oxygen concentration for

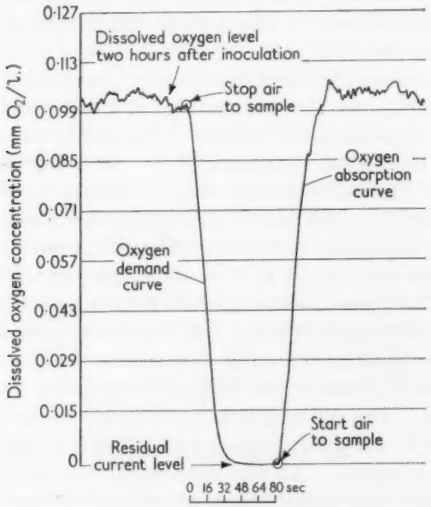

Fig. 4. Typical oxygen demand polarograph record for *S. Marcescens*

the culture and approached the residual current level asymptotically. The culture was sampled with a sterile syringe through the gum rubber sampling adaptor in the fermentor wall. The sample was diluted and plated on nutrient dextrose agar for viable cell count. The oxygen demand rate per viable *S. marcescens* cell could be calculated. The oxygen absorption coefficient for the fermentor could be calculated by substitution of appropriate values in equations (1) and (2).

The polarograph was operated continuously during the propagation of the *S. marcescens* culture (usually six to ten hours).

At half-hour intervals, the polarographic sampling technique, as previously described, was used to measure oxygen demand by the culture and critical oxygen concentration. The culture was sampled at the same time for viable cell count so that the oxygen uptake per cell could be determined. During the periods between the oxygen demand samples, the medium dissolved oxygen concentration was continuously recorded.

Results

The saturated dissolved oxygen concentration was measured with the polarograph at 25°C in 0·5 M sodium chloride solution and compared with values determined by the Winkler method

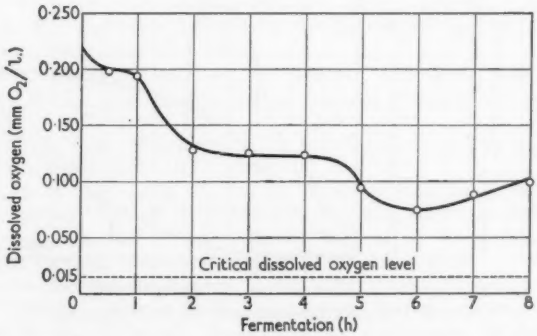

Fig. 5. Dissolved oxygen supply during propagation of *S. Marcescens*

and from Seidell[10] before each laboratory culture. The resultant standardization in millimoles of oxygen per litre per microampere was used to convert recorded current flow to dissolved oxygen concentration for the culture period. The standardization factor varied between 0·004 and 0·008 millimoles of oxygen per litre of solution per microampere, depending on the age and condition of the platinum and reference electrodes.

The dissolved oxygen concentration curve in Fig. 5 is the average for six runs with *S. marcescens* in the culture vessel at the operating conditions indicated previously. The minimum dissolved oxygen concentration during these observations with

S. marcescens was 0·075 millimoles of oxygen per litre of medium at six hours after inoculation of the culture.

The critical oxygen concentration for the *S. marcescens* culture is indicated in Fig. 5 at 0·015 millimoles of oxygen per litre.

The oxygen demand rate curves for *S. marcescens* culture and for individual viable cells in Fig. 6 are the average of six runs in the culture vessel at identical operating conditions. The maximum oxygen demand rate for the *S. marcescens* culture during these observations was 0·377 millimoles of oxygen per litre per minute at eight hours after inoculation of the culture. The maximum oxygen demand rate for *S. marcescens* cells was 0·5 ×

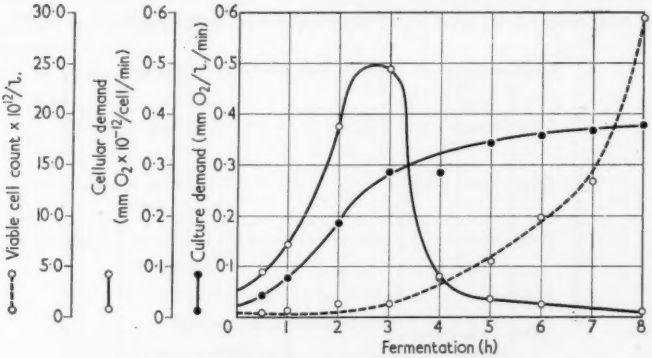

Fig. 6. Oxygen demand rates for *S. Marcescens*

10^{-12} millimoles of oxygen per cell per minute at three hours after inoculation. The oxygen demand rate by the growing culture, the dissolved oxygen concentration, and the critical dissolved oxygen concentration, may be obtained directly from the polarograph chart. The oxygen demand by an individual viable cell may be calculated from simultaneous samples and counts.

An average oxygen absorption coefficient of 2·06 per minute was determined by the 'gassing out' polarographic method for the culture system. The oxygen absorption coefficient for the culture system was calculated during the peak oxygen demand for the *S. marcescens* runs at 2·65 per minute. The coefficient indicates the oxygen-supplying ability of the aerobic culture system for the operating conditions utilized.

In this work, we have demonstrated that the respiration rate is highest for young *S. marcescens* cells in the early logarithmic growth phase and decreases with age. In young *S. marcescens* cultures, the total oxygen demand is low because of fewer viable cells. The maximum oxygen demand is reached at the end of the logarithmic growth phase when the product of viable cells and specific oxygen uptake is highest.[13]

It is evident that the sampling method of polarographic analysis could be applied to mass propagation of other aerobes in other media and to larger-scale equipment if the sample circulated through the polarographic cell is representative of the cultural conditions.

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Continuous Culture Experiments with *Saccharomyces rouxii* *†

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Summary. Growth of *Saccharomyces rouxii*, an osmophilic yeast that produces glycerol and arabitol, was investigated using chemically defined media in continuous culture. Total polyol production increased with increasing aeration and growth rate. At very low aeration levels ethanol was produced but as aeration levels were increased ethanol could not be detected, formation of *d*-arabitol remained constant and formation of glycerol increased.

In fully aerobic cultures with a replacement time of twelve hours, nitrogen-limited growth resulted in accumulation of glycerol in the medium. In glucose-limited growth glycerol tended to decrease. It was probably metabolized as a secondary carbon source. The optimum temperature for polyol production was 30°C while that for cell growth was 27°C.

Introduction

Recently, interest has been shown in the use of osmophilic yeasts for the production of glycerol and polyols. Nickerson and Carroll[9] demonstrated the production of glycerol by *Zygosaccharomyces acidifaciens* in addition to ethanol, carbon dioxide, lactic and acetic acids. Spencer and colleagues[14] investigated several species of *Zygosaccharomyces* and showed that these organisms were also capable of producing polyhydric alcohols. A species of yeast, *Saccharomyces rouxii* was studied in some detail[13] and shown to produce glycerol and *d*-arabitol in high yields. Peterson, Hendershot and Hajny[11] have since performed a survey of some eleven species in the genus Zygosaccharomyces for glycerol and *d*-arabitol production.

* Presented at the American Chemical Society meeting in Atlantic City, N.J. September, 1959.

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All this work was performed in batch culture experiments which used economic or semi-defined media.

The experiments to be described were performed with *Sacch. rouxii* grown in chemically defined media using the continuous culture technique originated by Monod [7] and Novick and Szilard.[10]

Apparatus

The apparatus is illustrated in Fig. 1. The culture vessel (fermentor), with a culture capacity of 600 ml, was constructed from a 2-l. Pyrex reaction kettle (Corning Glass, New York). Medium was introduced by a metering pump (D.C.L. Micropump—Distiller's Company Ltd., Great Burgh, Epsom, Surrey, England) from the medium reservoir and entered the culture vessel as a continuous drip feed; this displaced an equal volume of the culture from the overflow as the effluent product. Inside the culture vessel was a set of radial baffles which supported a central draught tube; a six-bladed impeller was driven in the vertical axis of this by a variable speed drive. A flow of filtered, sterile air at constant pressure was directed into the centre of the draught tube; the effluent gases left the culture vessel through a condenser, to remove the water vapour, and were metered by a flowmeter. The effluent culture was collected in a receiver and harvested at intervals. Fresh medium was added to the medium reservoir as required.

Samples of medium were obtained from the medium input line, and samples of culture were obtained from the overflow outlet, i.e. immediately before entering and immediately after leaving the culture vessel respectively. In some experiments the culture vessel was accommodated in a thermostatically controlled water bath and in others the whole apparatus was placed in a room at controlled temperature. Thermometers gave direct readings of the temperature of the culture inside the culture vessel.

The circulation of the agitated culture in the culture vessel gave complete foam control and excellent aeration: no antifoam was required, and values of 500 millimoles oxygen per litre per hour were obtained for measurements of Cu^{2+} catalysed sulphite oxidations made after the method of Cooper, Fernstrom and Miller.[2]

The apparatus was sterilized empty by autoclaving at 15 lb/in^2 for 30 min.

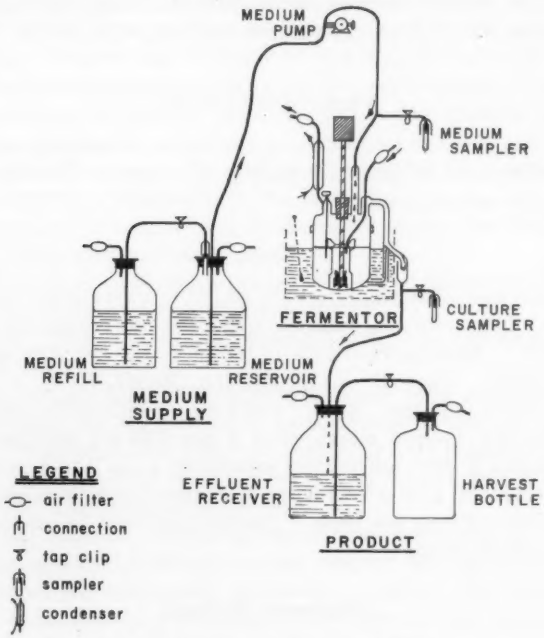

Fig. 1. Continuous culture apparatus

Materials and Methods

Unless otherwise stated, all the experiments were performed at 30°C.

Organism

The P_3a strain of *Sacch. rouxii* of Spencer [12] was used throughout these experiments. The culture was maintained on slants of a medium containing 60 per cent honey, 0·25 per cent yeast extract and 2 to 2·5 per cent agar. Inoculum was prepared by incubating the culture for 48 h at 30°C on a rotary shaker, in the medium to be used in the experiment: 10 per cent by volume was added to

250 ml of fresh medium in the culture vessel, and after a few hours' exponential growth the medium flow was started. In later experiments, the culture vessels were inoculated directly with a culture taken from the culture vessel from another experiment.

Purity of the Culture

This was checked by routine examination of samples under the microscope, and by periodic plating of suitable dilutions of the culture on various media.

Media

Glucose and $(NH_4)H_2PO_4$ were added in varying amounts to a medium of the following composition. Part A: contained glucose, KCl 0·43 g; $MgSO_4 \cdot 7H_2O$ 0·25 g; biotin 1·5 μg per 800 ml of distilled water. Part B: $(NH_4)H_2PO_4$; $ZnSO_4 \cdot 7H_2O$ 1·75 mg; $CuSO_4 \cdot 5H_2O$ 0·96 mg; $MnSO_4$ 0·005 mg; $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ 1·05 mg and sodium citrate 0·10 g per 220 ml distilled water. Parts A and B were autoclaved separately at 15 lb/in^2, and mixed after cooling to give a colourless transparent medium containing the above amounts per litre. This medium will be referred to as the simple medium.

Analytical Methods

Measurement of Growth. The cell growth was estimated by measuring the optical density of suitable dilutions of the culture in a Coleman Universal Spectrophotometer at 600 mμ. The instrument was calibrated against frequent determinations of dry weight of the organisms.

Dry Weight of Cells. 10 to 15 ml of the culture was centrifuged at 4,000 rev/min for 15 min and after decanting off the supernatant, the residue was washed twice with distilled water, followed by a final washing with methyl alcohol and then dried at 80°C overnight in a vacuum oven.

pH. Determinations were made using a Pye Model 605 pH meter.

Chromatography. (*a*) Paper chromatograms were used for routine examination of the culture. Ascending chromatograms were

developed with butanol–acetic acid–water solution (5 : 1 : 2, single phase) according to Hough[6]; or by an ethylacetate–glacial acetic acid–water mixture of 9 : 2 : 2. The reducing sugars, polyhydric alcohols and similar compounds were detected by the method of Trevelyan, Proctor, and Harrison [15] with alkaline silver nitrate.

(*b*) Partition chromatography on Celite–water columns was used for separations of glycerol from arabitol, using benzene–butanol mixtures as solvents.

Inorganic Phosphate. The inorganic phosphate was determined by the method of Fiske and Subba Row.[3]

Ammonia. Ammonia was determined using the method of Conway[1] in a microdiffusion cell.

The following analytical procedures were performed by the methods of Neish[8] unless otherwise stated.

Ethanol. Ethanol was determined using the Conway microdiffusion technique.

Polyhydric alcohols. Polyhydric alcohols were determined by per-iodic acid oxidation followed by colorimetric estimation of the formaldehyde produced with chromotropic acid reagent. When glycerol and *d*-arabitol were not separately determined, the total polyol yield was estimated as glycerol.

Reducing Sugars. Reducing sugars were estimated as glucose, using the modified Somogyi copper reagent and measuring the colour developed with arsenomolybdate reagent. The method of alkaline reduction of potassium ferricyanide in the presence of KCN, after Hoffmann,[5] was also used.

Organic Acids. Organic acids were determined by ether extraction followed by partition chromatography on a silica–water column.

Results

Growth on Simple Chemically Defined Media

A chemically defined medium was desirable, and that of Hendershot[4] was suitable when simplified. Biotin was the only growth factor necessary for rapid growth of the organism and addition of 1·5 μg/l. satisfactorily replaced yeast extract or corn steep liquor additions. The respective media are listed in Table Ia, and Table Ib gives the data obtained in an experiment using

rotary shake flasks to compare the growths of *Sacch. rouxii* obtained on these.

Table I(a). Chemically defined media used for growth of *Sacch. rouxii*

Constituent	(a) Hendershot*	(b) Modified medium	(c) + Yeast extract
Glucose	100 g	100 g	100 g
$(NH_4)H_2PO_4$	6·0 g	6·0 g	6·0 g
KH_2PO_4	0·2 g	0·2 g	0·2 g
KCl	0·43 g	0·43 g	0·43 g
$MgSO_4 \cdot 7H_2O$	0·25 g	0·25 g	0·25 g
Sodium citrate	1·00 g	0·10 g	0·10 g
l-Asparagine	2·50 g	2·50 g	2·50 g
$ZnSO_4 \cdot 7H_2O$	1·75 mg	1·75 mg	1·75 mg
$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$	1·05 mg	1·05 mg	1·05 mg
$CuSO_4 \cdot 5H_2O$	0·096 mg	0·096 mg	0·096 mg
$MnSO_4 \cdot H_2O$	0·005 mg	0·005 mg	0·005 mg
Biotin	0·01 mg	0·0015 mg	
Calcium pantothenate	0·50 mg	—	
Inositol	10·00 mg	—	replaced by 1 g/l.
Nicotinic acid	2·00 mg	—	Difco yeast ex-
Thiamine HCl	1·00 mg	—	tract
Pyridoxamine HCl	1·00 mg	—	
Distilled water	to 1 litre	to 1 litre	to 1 litre
Sterilization	15 min at 121°C	15 min at 121°C	15 min at 121°C

* *M.Sc. Thesis*, University of Wisconsin (1956)

Table I(b). Growth of *Sacch. rouxii* in rotary shake flask culture on defined media

Medium	Fermentation time, h	Dry wt. of cells, mg/ml	Final glucose conc., mg/ml	Chromatography			Final pH
				glycerol	arabitol	glucose	
(a)	80	4·15	None	+	+	−	3·00
(b)	80	4·05	Trace	+	+	+	2·95
(c)	75	4·75	None	+	+	−	3·20

Gump rotary shaker: 230 oscillations per min, radius 1 in.
Temperature 28°C
120 ml medium in 500-ml Erlenmeyer flask
20 ml washed cell suspension as inoculum

When modified medium (b)—Table Ia—was used in the continuous culture vessel on batch culture, the growth curve shown in Fig. 2 was obtained. The growth constants calculated from this

curve were yield constant (Y_{gl}) = 0·33 and a value of 8·5 h for the mean generation time of the organism.

The results obtained with a continuous flow of medium are given by Fig. 3, which shows the cell concentration and cell output plotted against the dilution rate. Steady states were obtained at replacement times above 8 h, and for optimal growth above

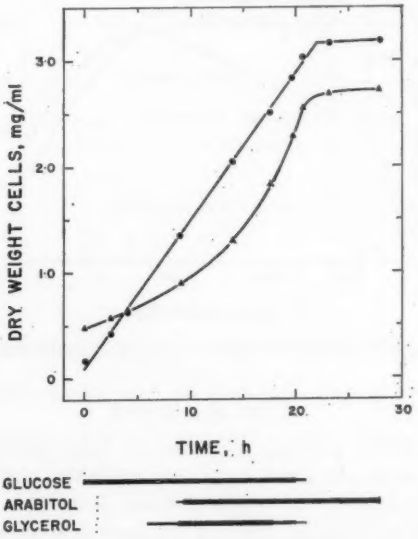

Fig. 2. Growth of *Saccb. rouxii* in batch culture: showing dry weight of cells and semi-log plot, utilization of glucose and formation of glycerol and arabitol

about 11 h. The latter value corresponds to a doubling time of 7·5 h, compared with 8·5 h obtained in the batch experiment. The maximum production rate, as cell output, was about 10 h.

These results were obtained by running both batch and continuous growths in the same reactor, i.e. under identical conditions, apart from that of medium supply. This is necessary for accurate comparison; experiments which use shake flasks for batch and fermentors for continuous growth comparisons are of limited use and often show divergences in the results which render the

comparisons useless. Shake flask experiments are useful for comparisons between themselves, e.g. evaluation of media etc., but not with fermentors. Some reasons for this can be seen in the following experiment.

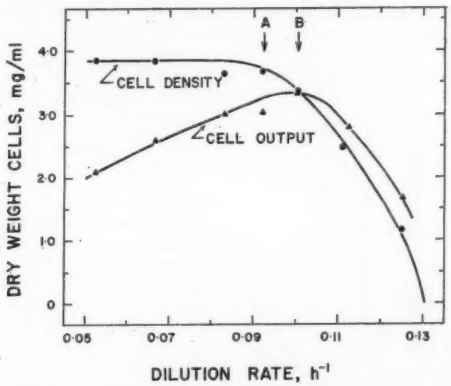

Fig. 3. Growth of *Sacch. rouxii* in continuous culture

Effect of Aeration

Microaerophilic, partially aerobic or fully aerobic conditions for growth were obtained by using suitable airflow rates and stirring conditions in the culture vessel (see Table II).

The degree of aeration affected the growth of *Sacch. rouxii* P_3a, when the organism was grown in the simple medium containing

Table II. Culture vessel—conditions of aeration

Condition	Air flow (l./l. culture/min)	Impeller* (rev/min)	Oxygen absorption coefficient† millimoles oxygen per litre per hour
I	None (free diffusion)	600	below 5
II	0–20	900	25–150
III	20–40	1475	250–500

I—microaerophilic; II—partially aerobic; III—fully aerobic
Temperature 30°C
* Impeller had 6 radial blades 1 in. × 1 in. and revolved with $\frac{1}{4}$-in. clearance in baffle.
† Cu^{2+} catalysed sulphite oxidation values after Cooper *et al.*[2]

glucose (10 g/l.) and $(NH_4)H_2PO_4$ (6 g/l.). Fig. 4 illustrates the growth curves and Table III summarizes some of the differences found under the above conditions.

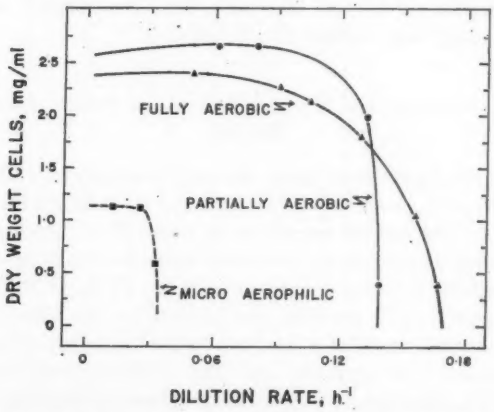

Fig. 4. Growth of *Sacch. rouxii* in continuous culture, effect of aeration

Table III. Continuous culture of *Sacch. rouxii*—effect of different rates of aeration

Condition	Dilution rate,* h^{-1}	Replacement time,* h	Y_{g1}†	Ethanol	Chromatography			pH range
					glycerol	arabitol	glucose	
I	0·03	33	—	++	+	+	+	3·0–3·5
	0·035	28·5						
II	0·11	9·1	0·30	±	+	+	+	2·8–3·2
	0·14	7·1						
III	0·13	7·7	0·27	−	++	+		2·4–2·8
	0·17	5·9						

Temperature 30°C
Medium: simple medium containing 10 g/l. glucose, 6 g/l. $(NH_4)H_2PO_4$
* The upper value is maximal, the lower one is critical.
† Y_{g1} denotes dry wt. cells/glucose used.

An increase in the growth rate occurred with increase in rate of aeration. Ethanol was not detected during fully aerobic growth, but it gradually increased in amount as the rate of aeration decreased. Formation of *d*-arabitol and glycerol was

followed by paper chromatography and showed a general constancy of arabitol production but wide variation in glycerol formation; the total polyol values determined generally increased with increased aeration rate and with longer growth rates. In microaerophilic conditions the cells were vacuolated, otherwise the morphology was similar in all cases.

Effect of Carbon and Nitrogen Sources as Growth Limiting Factors

Medium changes were made in one constituent $(NH_4)H_2SO_4$ (0·25 to 6·0 g/l.), in an experiment run at a standard replacement rate of 12 h. The results are given in Table IV. During this and the following experiments, aeration was non-limiting and there was negligible ethanol production. At $(NH_4)H_2PO_4$ concentrations below 1 g/l., growth was limited by the nitrogen source and glycerol and arabitol were both formed roughly in proportion to the amount of growth. At the highest concentration of $(NH_4)H_2PO_4$, the carbon source had become the limiting condition with a decrease in the total polyol yield. The yield constants, calculated in terms of glucose uptake (Y_{gl}), were approximately constant throughout. The yield constant, calculated in terms of the nitrogen uptake (Y_N), showed a change in value from nitrogen limited to nitrogen non-limited growth, and there was an increased uptake of nitrogen from the medium during the latter phase which did not materialize as increased cell growth. Phosphate uptake was insignificant at all stages and no changes were observed when the phosphate concentration was decreased by elimination of KH_2PO_4 from the medium.

In another experiment, growth was obtained in media containing fixed concentrations of $(NH_4)H_2PO_4$ (2 g/l.) and varying amounts of glucose (10 to 80 g/l.): again the standard replacement time was 12 h. The results are summarized by the data given in Table V.

The total polyol production remained fairly constant, though arabitol decreased and glycerol increased with increasing glucose concentration. The increase in cell yield, decrease in yield constant (Y_{gl}) and the constant uptake of nitrogen suggest an incorporation of glucose into the cells probably as carbohydrate.

Table IV. Continuous culture of *Sacch. rouxii*. Effect of varying concentrations of carbon and nitrogen source on steady state

Glucose, mg/ml	$(NH_4)H_2PO_4$, mg/ml	Dry wt. cells, mg/ml	Glucose used, mg/ml	Y_{gl}	Y_N	Total polyol, mg/ml	Ethanol, mg/ml	Chromatography			pH
								Gl	A	G	
10	6·0	2·50	9·6	0·26	0·16	1·55	0·0	?	+	+	2·6
10	2·0	1·50	6·0	0·25	0·16	2·75	0·08	+	+	+	2·55
10	1·0	1·64	7·3	0·23	0·23	3·20	0·015	+	+	+	2·50
10	0·5	1·30	5·3	0·25	0·20	1·40	0·00	+	+	+	2·40
10	0·25	0·73	2·8	0·26	0·21	0·90	0·00	+	+	+	2·75

Temperature 30°C.
Replacement time 12 h.
Medium: simple medium supplemented as in table.

Table V. Continuous culture of *Sacch. rouxii*. Effect of increasing concentrations of glucose on nitrogen-limited growth

$(NH_4)H_2PO_4$, mg/ml	Glucose, mg/ml	Dry wt. cells, mg/ml	Glucose used, mg/ml	Total polyol, mg/ml	Ethanol, mg/ml	Y_{gl}	Y_N	Chromatography			pH
								Gl	A	G	
2	10·0	1·50	6·0	2·7	0·00	0·25	0·16	+	+	+	2·55
2	20·0	1·68	6·9	2·4	0·20	0·24	—	+	+	+	2·45
2	40·0	1·80	8·8	2·9	—	0·20	0·19	+	·:·	+	2·40
2	80·0	1·88	10·6	3·0	0·60	0·18	0·22	+	?	+	2·45

Temperature 30°C.
Replacement time 12 h.
Medium: simple medium supplemented as in table.

An increase in the iodine staining properties of the cells and an increased value for cell carbon determinations in the 4 per cent and 8 per cent media supported this possibility. There was some ethanol formation at the higher sugar concentration, though conditions were not considered to have been oxygen-limited.

Effect of Temperature

In a short experiment, the effect of temperature on the growth of P_3a in the simple medium containing 80 g/l. glucose and 2 g/l. $(NH_4)H_2PO_4$ was determined at 27°, 30° and 35°C. Optimal polyol formation was found at 30°, but the amount of growth was greatest at 27° (see Table VI).

Table VI. Continuous culture of *Sacch. rouxii*. Effect of temperature on the steady state

Temp., °C	Dry wt. cells, mg/ml	Glucose used, mg/ml	Polyol, mg/ml	Chromatography			pH
				Gl	A	G	
27	2·80	37	2·65	+	+	+	2·35
30	2·50	25	6·10	+	+	+	2·20
35	1·12	20	3·60	+	+	+	2·70

Replacement time 15 h.
Medium: simple medium containing 80 g/l. glucose, 2 g/l. $(NH_4)H_2PO_4$.

Discussion

Using continuous culture techniques for the study of growth processes, it is essential to define the conditions of growth. The use of natural growth supplements, i.e. yeast extract or corn steep liquor, though useful economically for speeding up growth and increasing yields, also introduces miscellaneous carbon and nitrogen sources and other extraneous factors which obstruct and confuse the experimental results. For this reason a chemically defined medium was used, and this was simplified as far as possible.

The importance of aeration in polyol fermentations has been established by other workers. It was shown to affect the growth rate besides influencing the relative yields of the various products. In the majority of the experiments described, fully

aerated conditions, i.e. non-limiting for oxygen, were considered to exist in the culture: sulphite oxidation values were of the order 250 to 500 millimoles oxygen per litre per hour. Pirt[12] has given a value of 0·4 for the oxygen demand constant (the mole oxygen demand per mole substrate carbon utilized) for yeast grown aerobically in glucose medium. When this value was used to calculate the maximum oxygen demand required in the above experiments (i.e. 80 g/l. glucose at $D = 0{\cdot}15$ h^{-1}) a value of 160 millimoles oxygen per litre per hour was obtained, assuming the glucose to be completely oxidized to carbon dioxide. The experimental results were much lower than this, and the oxygen demand of the cultures was generally about 0·1 of this calculated value, i.e. well below the available capacity of the reactor. Thus the appearance of ethanol in the later experiments can hardly be interpreted as being due to a lack of oxygen; however, as no direct experimental determinations of oxygen uptake were made in these experiments, no conclusions can be drawn.

The nature of the factor controlling growth appeared to be significant in determining the product formation in the culture. In aerobic conditions, when nitrogen-limited growth was used, i.e. glucose non-limiting, glycerol tended to accumulate, but in glucose-limited growth glycerol tended to decrease—probably being catabolized as a secondary carbon source. There was also a tendency for nitrogen-limited cultures to grow at pH levels lower than in glucose-limited cultures, and when high concentrations of glucose were used the pH was sometimes as low as 2·3 due to succinic and acetic acid formation. Further variations in product formation occurred when different temperatures were used for growing the culture.

Following a continuous culture through a range of different flow rates can be relatively time consuming and the use of a standard replacement time reduced the time required to make comparisons of *closely related* media. It was found that such media gave similar growth curves and the method was acceptable for limited medium changes: the practice could not be recommended for studies with widely different media, however. The value of twelve hours was chosen for the standard replacement time used as that at which optimal growth was attained, i.e. the point where the concentration of the growth-limiting factor had

become minimal; it was also close to the point of optimal cell production.

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